ORIGINAL SUBMISSION

House Wellness Foods Corporation

IMOJI 3-20, ITAMI, HYOGO, 664−0011, JAPAN

SENT *VIA* FEDEX

January 29, 2010

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notice for a Heat-Killed *Lactobacillus plantarum* ingredient

Dear Dr. Martin:

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the notifier [House Wellness Foods Corporation (HWFC), Imoji 3-20, Itami, Hyogo, 664-0011, Japan], a Notice of the determination, on the basis of scientific procedures, that HWFC's heat-killed *Lactobacillus plantarum* ingredient, as defined in the enclosed documents, is GRAS under specific conditions of use as a food ingredient, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, which includes a comprehensive summary of the data available and reviewed by an independent panel of experts in support of the safety of the heat-killed *L. plantarum* ingredient under the intended conditions of use, also are enclosed.

I trust that the enclosed Notice is acceptable. Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Tetsuya Matsumoto
House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo
664-0011
Japan

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ハウス ウェルネス フーズ 株式会社 〒664-0011 兵庫県伊丹市鋳物師三丁目20番地

GRAS Exemption Claim for a Heat-Killed *Lactobacillus plantarum* (HK-LP) Ingredient

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by:

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo
664-0011
Japan

January 11, 2010

GRAS Exemption Claim for a Heat-Killed *Lactobacillus plantarum* (HK-LP) Ingredient

Table of Contents

List of Appendices

List of Figures and Tables

000006

GRAS Exemption Claim for a Heat-Killed *Lactobacillus plantarum* (HK-LP) Ingredient

I. GRAS EXEMPTION CLAIM

A. Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

A heat-killed *Lactobacillus plantarum* (HK-LP) ingredient, comprising 20% HK-LP strain L-137 and 80% dextrin, has been determined by House Wellness Foods Corporation (hereafter HWFC) to be Generally Recognized as Safe (GRAS) for use in a variety of traditional food products, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use in food. Therefore, the use of the HK-LP ingredient in food as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Jan. 8, 2010
Date

Tetsuya Matsumoto
General Manager
International Department
House Wellness Foods Corporation

B. Name and Address of Notifier

Tetsuya Matsumoto
General Manager
International Department
House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo
664-0011
Japan

Telephone: +81-(0)72-778-1195
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Email: Matsumoto_Tetsuya@house-wf.co.jp

C. Common Name of the Notified Substance

Heat-killed *Lactobacillus plantarum* (HK-LP) ingredient.

D. Conditions of Intended Use in Food

HWFC intends to market the HK-LP ingredient (trade name LP20), comprising 20% HK-LP and 80% dextrin, as a food ingredient in the United States (U.S.) in a variety of traditional food products, including baked goods and baking mixes, beverages and beverage bases, breakfast cereals, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk and milk products, plant protein products, processed fruit and fruit juices, processed vegetables and vegetable juices, soft candy, soups and soup mixes, and sugar substitutes at a maximum level of 150 mg per serving. The maximum use level of 150 mg of the final ingredient per serving provides approximately $3x10^{10}$ HK-LP cells per serving.

E. Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) § 170.30, the HK-LP ingredient has been determined by HWFC to be GRAS on the basis of scientific procedures (U.S. FDA, 2009). This GRAS determination is based on data generally available in the public domain pertaining to the safety of HK-LP and *L. plantarum*, as discussed herein, and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of the HK-LP ingredient as a component of food [see Appendix A, entitled, "**EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF A HEAT-KILLED *LACTOBACILLUS PLANTARUM* INGREDIENT FOR USE IN FOODS**"].

At the request of HWFC, an Expert Panel ("the Expert Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened on February 19, 2009 to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether the intended uses of the HK-LP ingredient as a food ingredient are safe and suitable and would be GRAS based on scientific procedures.

The Panel consisted of the following qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin), Stephen L. Taylor, Ph.D. (University of Nebraska), and Gary M. Williams, M.D. (New York Medical College).

The Expert Panel convened on behalf of HWFC, independently and collectively, and critically evaluated the data and information summarized herein and concluded that the intended uses in traditional foods described herein for the HK-LP ingredient, meeting appropriate food-grade specifications and manufactured according to current Good Manufacturing Practice (cGMP), are

safe and suitable and GRAS based on scientific procedures. It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion.

The HK-LP ingredient is GRAS based on scientific procedures for its intended use as a food ingredient; therefore, it is excluded from the definition of a food additive and thus may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

F. Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo
664-0011
Japan

Should the FDA have any questions or additional information requests regarding this notification, HWFC will supply these data and information.

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

A. Identity

The HK-LP ingredient comprises 20% HK-LP and 80% dextrin. The HK-LP, although heat-killed, is not lysed and broken down during heat treatment, but maintains the structural form of intact live bacteria. The ingredient is a light brown powder free of unfavorable organoleptic properties, such as off-taste and off-flavor.

HWFC's HK-LP ingredient is prepared from a stock culture of *L. plantarum* strain L-137, which was originally isolated and identified from a fermented rice and fish dish called "burong isda" (for "fermented fish") that is native to the Philippines (Olympia *et al.*, 1992, 1995). *L. plantarum* L-137 is a gram-positive, rod-shaped, non-motile, lactic acid-producing bacterium (Olympia *et al.*, 1992). The taxonomic identification of the organism was determined by morphological and physiological analyses, as well as *via* DNA-DNA hybridization assays using type strains of various *Lactobacillus* species, including *L. plantarum* [obtained from Japan Collection of Microorganisms (Accession No. 1149); Waiko, Saitama, Japan], as reference strains as described in Olympia *et al.* (1992). HWFC has deposited *L. plantarum* L-137 in the National Institute of Advanced Industrial Science and Technology's International Patent Organism Depository (deposit FERM BP-08607). HWFC maintains quality control procedures to ensure that the strain used in the manufacture of HK-LP is *L. plantarum* L-137. The identity of the strain is confirmed as *L. plantarum* L-137 in each batch of the ingredient using an enzyme-linked immunosorbent assay (ELISA) with the use of an established monoclonal antibody (1A11), which selectively recognizes *L. plantarum* strain L-137. A summary of the current taxonomic assignment for *L. plantarum* L-137 is presented in Table II.A-1.

Table II.A-1 Taxonomic Assignment: *Lactobacillus plantarum*

Taxonomy	Taxonomic Assignment
Kingdom	Bacteria
Division	*Firmicutes*
Class	*Bacilli*
Order	*Lactobacillales*
Family	*Lactobacillaceae*
Genus	*Lactobacillus*
Species	*Lactobacillus plantarum*
Strain	*Lactobacillus plantarum* L-137

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B. Method of Manufacture

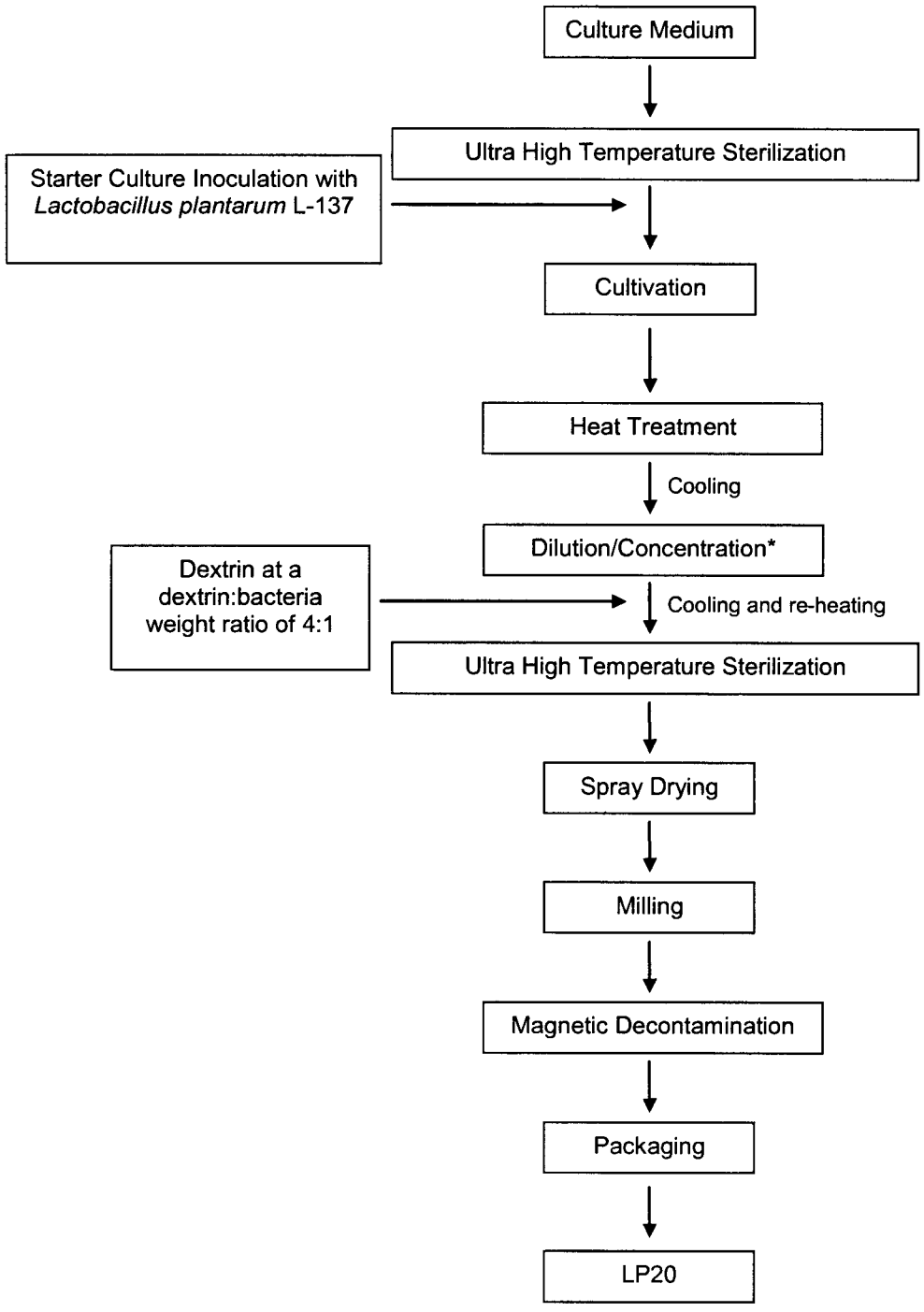
A schematic diagram of the general manufacturing process employed to produce the HK-LP ingredient is illustrated in Figure II.B-1. The first step in the manufacturing process involves fermentation of a starter culture of *L. plantarum* L-137 using a food-grade culture medium, consisting of skim milk hydrolysate (containing lactose and hydrolysates of the bovine milk proteins casein and β-lactoglobulin), yeast extract, maltose, polyoxyethylene sorbitan monooleate, manganese sulfate, sodium acetate, and sodium hydroxide. Prior to addition of the bacteria, the pH of the culture medium is adjusted from pH 6.8 to 7.1 with the addition of an aqueous 25% sodium hydroxide solution. The medium is then sterilized at 121°C for 15 minutes and cooled to 32°C. The medium is inoculated with *L. plantarum* L-137 and the bacteria are cultured for up to 24 hours.

Additional medium is prepared in a mother tank and heated to 50 to 60°C with stirring. The pH of the medium is adjusted from 6.8 to 7.1 with the addition of 20% aqueous sodium hydroxide, at which point the culture medium undergoes preliminary sterilization at 80°C and is then cooled to 50 to 55°C. This culture medium is transported to another tank and flushed with water prior to ultra high temperature (UHT) sterilization at 125 to 135°C for 5 seconds. The medium is cooled to 30 to 35°C and then inoculated with the starter culture. The bacteria are cultured over a period of up to 24 hours at this temperature, following which the bacteria are killed by heat-treatment until the medium temperature reaches 80°C. The medium containing HK-LP is cooled to a temperature of 15 to 20°C using chilled water, and is subsequently filtered with a microfiltration membrane to remove the medium components and to concentrate the HK-LP. The HK-LP, although heat-killed, maintains the form of intact bacteria, and therefore, does not filter through the microfiltration membrane. The medium containing the HK-LP is subjected to 75 rounds of dilution with water and concentration by filtration, followed by a final filtration step, completing the dilution/concentration process. During the dilution/concentration process, the culture medium is diluted up to $1x10^6$-fold. The HK-LP solution is transported to a storage tank, flushed with water, and adjusted for fluid volume. A sample is taken to measure the bacterial weight using the loss-on-drying method. The HK-LP solution is cooled to 5 to 8°C using chilled water and then heated to 50 to 60°C. Dextrin is then added to the HK-LP solution, for formulation purposes, at a dextrin to bacteria weight ratio of 4:1 and filtered with the use of a filter cloth. This latter filtration step removes any aggregated HK-LP, undissolved dextrin, or foreign substance contamination, ensuring clarity of the HK-LP product prior to spray drying. In the final steps of the manufacturing process, the product is UHT-sterilized at 120 to 122°C for 10 seconds, cooled to 50 to 55°C, spray dried, milled, and freed of magnetic contamination prior to packaging. The final HK-LP ingredient comprises 20% HK-LP and 80% dextrin. All processing aids used in the manufacture of the HK-LP ingredient are used in compliance with appropriate federal regulations as indicated in Table II.B-1.

Table II.B-1 List of Processing Aids Used in the Manufacture of the Heat-Killed *Lactobacillus plantarum* ingredient

Processing Aids	Function of Processing Aid	Reference to Appropriate Use in Food
Sodium hydroxide (NaOH)	pH control agent	21 CFR § 184.1763 Sodium hydroxide
Microfiltration membrane (polysulfone type)	Filtration	21 CFR §177.1655 Polysulfone resins
Filter cloth (cotton fiber type)	Filtration	21 CFR §177.2260 Filters, resin-bonded
Dextrin	Formulation aid	21 CFR §184.1277 Dextrin

Figure II.B-1 Schematic Overview of the Manufacturing Process for the Heat-Killed *Lactobacillus plantarum* ingredient



* using a microfiltration membrane

C. Specifications and Analytical Data for Food Grade Material

The HK-LP ingredient is produced in accordance with cGMP, and in order to ensure a consistent and safe product, HWFC has established food-grade specification parameters for the final ingredient. These parameters comprise specifications for the physical appearance, identification, and purity of the HK-LP ingredient, as well as specifications for potential chemical and microbiological impurities and contaminants.

Approximately 20% (±2%) of the final product consists of HK-LP and approximately 80% is composed of dextrin. Moisture accounts for less than 8% of the final product composition. Specifications for residual levels of selected culture medium components also are included, and several impurity and microbiological specifications similar to those included for other food ingredients have been specified to ensure safety of the use of the ingredient in food. The number of live *L. plantarum* is included in the specifications to ensure that the manufacturing process incorporates sufficient methods to kill the bacteria component of the ingredient. Details of the methods of analyses are provided in Appendix B and the product specifications for the HK-LP ingredient are presented in Table II.C-1 and in Appendix C.

Analyses of three non-consecutive lots of the HK-LP ingredient confirm that the material produced by the manufacturing process is consistent and complies with the product specifications, meeting appropriate food-grade specifications. The analytical data also demonstrate the absence of any chemical impurities or microbiological contamination. Furthermore, other analytical data generated by HWFC confirm that residual levels of the raw materials used as components of the culture medium are well below the specification parameters, demonstrating successful removal of these materials during the filtration process. Inclusive in these analyses are analytical data for the major known milk allergens, β-lactoglobulin and casein, demonstrating levels of potential milk allergens below specified levels in the final ingredient as assessed using ELISA. The product also was analyzed for protein and nucleic acid content, resulting from the presence of HK-LP, and for the potentially harmful biogenic amines histamine and tyramine, which may be metabolically produced by lactobacilli. The analytical results demonstrate that neither histamine nor tyramine was detected in the HK-LP ingredient as assessed using high-performance liquid chromatography (HPLC). The complete certificates of analysis for these lots and other analytical data generated are provided in Appendix C.

Table II.C-1 Specifications for the Heat-Killed *Lactobacillus plantarum* Ingredient

Specification Parameter	Specification	Method of Analysis
Appearance	Light brown powder free of off-taste and off-flavor	Visual inspection and sensory test
Foreign substances	Negative	Visual inspection
HK-LP content	20±2%	Dry weight method for bacterial cell mass[a]
Dextrin content	76±6%	Glucose oxidase-mutarotase method following HCl hydrolysis[a]
Grain size (residue of #30)	0%	Standard sieve method with sieve opening of 500 µm[b]
Bovine milk protein	≤20 µg/g	ELISA[d]
Casein	≤10 µg/g	ELISA[d]
β-Lactoglobulin	≤10 µg/g	ELISA[e]
Lactose	≤2 mg/g	HPLC[f]
Loss on drying	≤8%	Loss on drying test[c]
Residue on ignition	≤30 mg/g	Residue on ignition[c]
Sodium acetate	≤100 µg/g	HPLC[f]
Manganese	≤30 µg/g	ICP emission spectrometry[c]
Lead	≤1 µg/g	Atomic absorption spectrophotometry[c]
Number of mesophilic aerobic bacteria	≤$1x10^3$ CFU/g	Plate count method[g]
Number of viable *L. plantarum*	≤$1x10^2$ CFU/g	Viable count determination[a]
Salmonella species	Negative	Isolation culture method[g]
Coliforms (including *Escherichia coli*)	Negative	Most probable number method[g]
Yeast and mold	≤$1x10^2$ CFU/g	Dilution plating method[g]

Abbreviations: As_2O_3 = arsenic oxide; CFU = colony-forming units; ELISA = enzyme-linked immunosorbent assay; HCl = hydrochloric acid; HK-LP = heat-killed *Lactobacillus plantarum*; HPLC = high-performance liquid chromatography; ICP = inductively coupled plasma; Pb = lead

[a] House Wellness Foods Corporation in-house method.
[b] Japanese Industrial Standards Z 8801.
[c] Japan's Specifications and Standards for Food Additives 7th Edition (2000).
[d] Ministry of Health, Labour and Welfare. 2006. Public Notification No. 0622003. Test Methods for Food Products Containing Allergic Substances. Ministry of Health, Labour and Welfare, Department of Food Safety, Pharmaceutical and Food Safety Bureau; Tokyo, Japan.
[e] Using Morinaga FASPEK Milk Measurement Kit (β-lactoglobulin), Morinaga Institute of Biological Science, Inc., Japan.
[f] Referee laboratory in-house method.
[g] Japan's Food Sanitation Inspection Guidance.

D. Stability of the HK-LP Ingredient

The stability of the HK-LP ingredient is characterized by the stability of the HK-LP component of the ingredient. Bulk ingredient stability data indicate that the number of HK-LP cells in the ingredient is stable for up to 16 weeks at 40°C, which empirically corresponds to approximately 2 years of storage at ambient temperatures. Additionally, from these data, the number of HK-LP cells per one gram of the final ingredient is an estimated 2.3×10^{11} cells. The results of further stability studies indicate that the HK-LP ingredient is expected to be stable when added to in acidic beverage formulations and low-moisture foods under appropriate storage conditions. When the ingredient is added to high-moisture foods (*e.g.*, tofu), the storage conditions, including temperature and appropriate expiration dates, is determined for each individual food as the stability of the ingredient may vary with moisture. See Appendix D for details on product stability.

III. SELF-LIMITING LEVELS OF USE

No self-limiting levels of use were identified for the HK-LP ingredient.

IV. BASIS FOR GRAS DETERMINATION

A. Documentation to Support the Safety of the HK-LP Ingredient

The determination that the HK-LP ingredient is GRAS is on the basis of scientific procedures, and the information supporting the general recognition of the safe use of the HK-LP ingredient includes:

- Background consumption of *L. plantarum* as added or natural components of various foods;
- The recognized history of safe consumption of *L. plantarum* in humans
- data pertaining to the identity, intended use, and estimated intake of the HK-LP ingredient;
- the expected metabolic fate of HK-LP; and
- preclinical and human studies assessing the safety of the HK-LP ingredient and live preparations of *L. plantarum.*

Moreover, these data were reviewed by a panel of experts, qualified by scientific training and experience to evaluate the safety of ingredients as components of food, who concluded that the intended uses of the HK-LP ingredient are safe and suitable and would be GRAS based on scientific procedures [see Appendix A, entitled, **"EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF A HEAT-KILLED *LACTOBACILLUS PLANTARUM* INGREDIENT FOR USE IN FOODS"**]. A summary of these data is presented herein.

B. Background Dietary Consumption and History of Safe consumption of *Lactobacillus plantarum* in Humans and Current Regulatory Status of Dextrin

B.1 Background Dietary Consumption of *Lactobacillus plantarum* in Humans

L. plantarum exists naturally in many different types of fermented foods and also is present in other food products as an ingredient. Traditionally, *L. plantarum* has been consumed as a component of fermented rice and fish dishes from the Southeast Asia region (Orillo and Pederson, 1968; Olympia *et al.*, 1992; Tanasupawat *et al.*, 1998). *L. plantarum* also is commonly consumed world-wide as a result of its occurrence in many other fermented foods, including, but not limited to, fermented vegetable dishes, pickles, sauerkraut, and in various types of cheeses and fermented sausages (Orillo *et al.*, 1969; Rebecchi *et al.*, 1998; Baruzzi *et al.*, 2000; Tamminen *et al.*, 2004; Rantsiou *et al.*, 2006, 2008; García Fontán *et al.*, 2007;

Plengvidhya *et al.*, 2007). As mentioned, *L. plantarum* L-137 was itself specifically isolated and identified from a fermented rice and fish dish called "burong isda" (for "fermented fish") that is native to the Philippines (Olympia *et al.*, 1992, 1995). Along with other bacterial species, yeasts, and molds, *L. plantarum* is responsible for the fermentation and ripening of such traditional foods.

In more recent history, *L. plantarum* strain 299v has been consumed as a constituent of a fermented oatmeal, fruit or yogurt drink under the name ProViva, which is marketed in parts of Europe (Molin, 2001), and has been subject to numerous human studies described in Section G. ProViva contains approximately $5x10^{10}$ colony forming units (CFU) of live *L. plantarum* 299v per liter, which is comparable in quantity to the number of HK-LP cells that would be provided from the intended uses of the HK-LP ingredient. The HK-LP ingredient itself has been marketed as a supplement ('encapsulated food') at a level of 52 mg/serving in Japan since 2005, and has been consumed as an ingredient in a Japanese food product (a granular-powder food product) at a level of 50 mg/serving since June 2008. The supplement and food use levels of the HK-LP ingredient provide approximately $1.2x10^{10}$ HK-LP cells per serving ($2.3x10^{11}$ HK-LP cells/g final ingredient). In addition, the HK-LP ingredient does not fall within the scope of the European Novel Foods Regulation as advised by the Food Standards Agency. This decision was based on a significant history of consumption of *L. plantarum* in Denmark prior to 15 May 1997. Furthermore, specific dietary consumption of dead lactobacilli occurs through consumption of yogurts that have been heat-treated following fermentation, a practice that is permitted by the FDA to destroy viable microorganisms (21 CFR §131.200, 21 CFR §131.203, and 21 CFR §131.206) (U.S. FDA, 2009).

Therefore, *L. plantarum* has a history of human consumption as a natural and added component of food. In addition, heat-killed lactobacilli have a safe history of human consumption in the U.S. as a result of heat treatment of yogurts. Furthermore, *L. plantarum*, along with other lactobacilli, is a commensal bacterium present as part of the normal intestinal and oral microflora of healthy individuals (Molin *et al.*, 1993; Ahrné *et al.*, 1998; Song *et al.*, 2000).

B.2 History of Safe Consumption of *Lactobacillus plantarum* in Humans

Species belonging to the *Lactobacillus* genus are gram-positive, non-pathogenic, non-spore-forming, and non-motile rods that occur ubiquitously (Bernardeau *et al.*, 2006). The history of use of lactobacilli in food and food production is generally accepted as safe by the scientific community as reviewed by a number of scientific groups and qualified experts (Adams and Marteau, 1995; Salminen *et al.*, 1998; Adams, 1999; Borriello *et al.*, 2003; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2006, 2008; Snydman, 2008); the details pertaining to the safety of lactobacilli and *L. plantarum* for human consumption, as discussed in these reviews, are summarized in Section D.

In addition, *L. plantarum* has been granted Qualified Presumption of Safety (QPS) status in the European Union by the European Food Safety Authority (EFSA). The QPS approach is a generic assessment system for use within EFSA that is applied in order to harmonize pre-market safety assessments of selected groups of microorganisms used in food and food production (EFSA, 2005). The QPS approach undertaken by the EFSA is similar in concept and purpose to the GRAS determination process of a food ingredient in that it is intended to give recognition and weight to the element of common knowledge of safety referred to as 'the body of knowledge' (EFSA, 2005). The determination of safety *via* the QPS approach is based on the following 4 pillars: identity (*i.e.*, taxonomic grouping), body of knowledge (including peer-reviewed scientific literature and public databases, history of use, industrial applications, clinical aspects, and ecology), possible pathogenicity, and end use (EFSA, 2007). Using the QPS approach, the EFSA Scientific Committee conducted an initial safety assessment to determine the suitability of species belonging to the genus *Lactobacillus* for QPS status (EFSA, 2007). The Scientific Committee noted that, although a variety of different *Lactobacillus* species, including *L. plantarum* have been occasionally isolated from human clinical specimens, such occurrences were rare and were chiefly encountered in immunocompromised patients or in patients with severe underlying illnesses. The Scientific Committee concluded that most *Lactobacillus* species can be considered to be non-pathogenic to humans, and therefore, there were no specific safety concerns regarding these species. Following their evaluation, the Scientific Committee granted a number of *Lactobacillus* species, including *L. plantarum*, QPS status based on the long history of safe use of these microorganisms in the food chain. Upon review in 2008, The Panel on Biological Hazards retained the QPS status of *L. plantarum* along with that of many other *Lactobacillus* species (EFSA, 2008).

Moreover, *L. plantarum* specifically appears on the inventory of microorganisms with a documented history of use in human food that was compiled by the International Dairy Federation in collaboration with the European Food and Feed Cultures Association (Mogensen *et al.*, 2002). The inventory lists microbial strains used by the food industry that have a long history of use in food without adverse effects. Also, according to conclusions of a workshop held by the Lactic Acid Bacteria Industrial Platform (LABIP) in the European Union, all lactobacilli should be considered as belonging to a no-risk category, with the exception of *L. rhamnosus* (Adams and Marteau, 1995).

B.3 Current Regulatory Status of Dextrin

Dextrin is used as a formulation aid in the production of the HK-LP ingredient. Dextrin is affirmed as GRAS by the FDA as a direct food substance with no limitation other than cGMP when used as a formulation aid, processing aid, stabilizer, thickener, or surface-finishing agent (21 CFR §184.1277) (U.S. FDA, 2009). Therefore, the dextrin component of the HK-LP ingredient does not pose any safety or regulatory issues.

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C. Estimated Intake of the HK-LP Ingredient

Estimates for the intake of the HK-LP ingredient were based on the intended food uses and use levels in conjunction with food consumption data included the National Center for Health Statistics' (NCHS) 2003-2004 National Health and Nutrition Examination Survey (NHANES) (CDC, 2006; USDA, 2009). The individual intended food uses and use levels of the HK-LP ingredient are presented in Table IV.C-1. Food codes representative of each intended food use were chosen from the 2003-2004 NHANES (CDC, 2006; USDA, 2009) and were grouped in food use categories according to Title 21, Section 170.3 of the CFR (U.S. FDA, 2009). Use levels were calculated based on the amount of the ingredient added to a serving of each individual food [*i.e.,* per Reference Amounts Customarily Consumed (RACC) per eating occasion, according to Section 101.12 of the CFR (U.S. FDA, 2009)]. Product-specific adjustment factors were developed based on data provided in the standard recipe file for the Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996, 1998 survey (USDA, 2000).

Table IV.C-1 Summary of the Individual Proposed Food Uses and Use Levels for a Heat-Killed *Lactobacillus plantarum* Ingredient in the United States

Food Category	Proposed Food Uses	Use Level (mg/RACC*)	RACC[a]	Use Level (%)
Baked Goods and Baking Mixes	Bagels	91.7	55 g	0.1667
	Quick Breads and Sweet Rolls	91.7	55 g	0.1667
	Yeast Breads and Rolls	83.3	50 g	0.1667
Beverages and Beverage Bases	Cocoa Drinks (Powder; Milk and Non-Milk based)	150.0	240 mL (reconstituted)	0.0625
	Energy, Sport, and Isotonic Beverages	61.0	240 mL	0.0254
	Enhanced and Bottled Water	61.0	240 mL	0.0254
	Fruit Drinks and Ades	61.0	240 mL	0.0254
	Non-Milk based Meal Replacements	61.0	240 mL	0.0254
	Soft Drinks	61.0	240 mL	0.0254
Breakfast Cereals	Ready to Eat Breakfast Cereals	45.0, 90.0, or 165.0	(Puffed) 15 g (Fiber) 30 g (Biscuit) 55 g	0.3000
Dairy Product Analogs	Soy Milk	150.0	240 mL	0.0625
	Coffee Whiteners	100.0	2 g	5.0000
Fats and Oils	Butter	75.0	15 g	0.5000
	Margarine	75.0	15 g	0.5000
Frozen Dairy Desserts	Frozen Yogurt	100.0	120 g	0.0833
Grain Products and Pastas	Cereal and Energy Bars (Grain-based Bars/Health Bars/Granola-type Bars, *etc.*)	100.0	40 g	0.2500
	Pizza Dough	165.0	55 g	0.3000

Table IV.C-1 Summary of the Individual Proposed Food Uses and Use Levels for a Heat-Killed *Lactobacillus plantarum* Ingredient in the United States

Food Category	Proposed Food Uses	Use Level (mg/RACC*)	RACC[a]	Use Level (%)
Milk and Milk Products	Drinkable Yogurt[b]	150.0	240 mL	0.0625
	Flavored Milk and Milk Drinks, Including Chocolate and Malt Drinks	150.0	240 mL	0.0625
	Fermented Milk[c]	150.0	240 mL	0.0625
	Milk-Based Meal Replacements	150.0	240 mL	0.0625
	Yogurt	187.0	225 g	0.0833
Plant Protein Products	Tofu	31.9	85 g	0.0375
	Tofu (Hamburgers and Steaks)	63.8	85 g	0.0750
	Soy based Bars	100.0	40 g	0.2500
Processed Fruits and Fruit Juices	Fruit Juices	150.0	240 mL	0.0625
	Nectars	150.0	240 mL	0.0625
Processed Vegetables and Vegetable Juices	Vegetable Juices	150.0	240 mL	0.0625
Soft Candy	Chocolate Confectionery	100.0	40 g	0.2500
Soups and Soup Mixes	Dry Soup Mixes, Including Miso and Miso Soup	1,225.0	245 g	0.5000
Sugar Substitutes	Table Top Sugar Substitutes	150	2	7.5000

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12 – U.S. FDA, 2009).
[b] Food codes representative of yogurt drinks were not identified. Fruit smoothie drinks were used as a surrogate code for yogurt drinks.
[c] Food codes representative of fermented milk were not identified. Milk with acidophilus and buttermilk were used as surrogate codes for fermented milk products.

The estimated intakes of the HK-LP ingredient from all intended food uses in the U.S. are presented in Tables IV.C-2 and IV.C-3 on an absolute and body weight basis, respectively. From the intakes analysis approximately 96.9% of the total U.S. population was identified as potential consumers of the ingredient from the intended food uses (8,007 actual users identified). Of all the population groups, female and male adults were determined to be the greatest percentage of users at 100%, followed by female and male teenagers and children at 99.9%. Infants were determined to be the lowest percentage of users at 72.6%. As shown in Tables IV.C-2 and IV.C-3, results for the all-person and all-user consumption estimates were essentially identical for most population groups as a result of the near 100% user percentages. The exception was the infant population group.

Table IV.C-2 Summary of the Estimated Daily Intake of a Heat-Killed *Lactobacillus* Ingredient from All Intended Food Uses in the United States by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (g)	90th Percentile (g)	Mean (g)	90th Percentile (g)
Infants	0 to 2	72.6	675	0.26	0.57	0.32	0.63
Children	3 to 11	99.9	1,286	0.49	0.81	0.49	0.81
Female Teenagers	12 to 19	99.9	991	0.50	0.92	0.50	0.92
Male Teenagers	12 to 19	99.9	998	0.67	1.18	0.67	1.18
Female Adults	20 and Up	100.0	2,128	0.62	1.21	0.62	1.21
Male Adults	20 and Up	100.0	1,929	0.71	1.31	0.71	1.31
Total Population	All Ages	96.9	8,007	0.61	1.14	0.62	1.14

Table IV.C-3 Summary of the Estimated Daily per Kilogram Body Weight Intake of a Heat-Killed *Lactobacillus* Ingredient from All Intended Food Uses in the United States by Population Group (2003-2004 NHANES Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-User Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infants	0 to 2	72.6	675	21.2	47.1	26.2	51.2
Children	3 to 11	99.9	1,286	18.5	32.7	18.5	32.7
Female Teenagers	12 to 19	99.9	991	8.8	16.0	8.8	16.0
Male Teenagers	12 to 19	99.9	998	10.5	18.5	10.5	18.5
Female Adults	20 and Up	100.0	2,128	8.6	17.0	8.6	17.0
Male Adults	20 and Up	100.0	1,929	8.5	16.2	8.5	16.2
Total Population	All Ages	96.9	8,007	10.5	21.4	10.6	21.6

For the total U.S. population, consumption of foods containing the HK-LP ingredient would result in estimated mean all-person and all-user intakes of the ingredient of 0.61 g/person/day (10.5 mg/kg body weight/day) and 0.62 g/person/day (10.6 mg/kg body weight/day), respectively (Tables IV.C-2 and IV.C-3). The 90th percentile all-person and all-user intakes of the ingredient by the total population from all intended food uses were estimated to be 1.14 g/person/day (21.6 mg/kg body weight/day). Based on an HK-LP content of 20% in the ingredient, the corresponding all-person and all-user mean and 90th percentile intakes of HK-LP were calculated to be 0.124 g/person/day (2.12 mg/kg body weight/day) and 0.228 g/person/day (4.32 mg/kg body weight/day), respectively. Therefore, the estimated mean and 90th percentile all-user intakes of HK-LP are approximately equivalent to an intake of 2.9×10^{10} and 5.2×10^{10} HK-LP cells/person/day, respectively.

On an individual population basis, the greatest mean all-person and all-user intakes of the HK-LP ingredient on an absolute basis were determined to be in male adults, at 0.71 g/person/day (Table IV.C-2). Infants were determined to have the lowest mean all-person and all-user intakes of the ingredient on an absolute basis, with values of 0.26 and 0.32 g/person/day, respectively. On a per kilogram body weight basis, mean all-person and all-user intakes of the ingredient were highest in infants with intakes of 21.2 and 26.2 mg/kg body weight/day, respectively (Table IV.C-3). The lowest all-person and all-user mean intake on a body weight basis was determined to be in male adults, with a value of 8.5 mg/kg body weight/day.

When heavy consumers (90^{th} percentile) were assessed on an absolute basis, all-person and all-user intakes of the HK-LP ingredient from all intended food uses also were determined to be greatest in male adults with a value of 1.31 g/person/day (Table IV.C-2). The lowest 90^{th} percentile all-person and all-user intakes were in infants, with value of 0.57 and 0.63 g/person/day. On a body weight basis, infants were determined to have the greatest all-user 90^{th} percentile intakes of the ingredient, with values of 47.1 and 51.2 mg/kg body weight/day, respectively (Table IV.C-3). The lowest all-user 90^{th} percentile intakes on a body weight basis were determined to be in female teenagers at 16.0 mg/kg body weight/day.

D. Special Safety Considerations Relevant to Microorganisms Proposed for Use in Food

Parameters that are relevant specifically to the safety of microorganisms proposed for use in food include taxonomy, pathogenicity, potential toxin production, antibiotic-resistance potential, metabolic considerations, environmental presence, and viability (Mattia and Merker, 2008). Also important is whether the microorganism is a common resident in the intestinal microflora and whether a history of safe use in food exists for the microorganism (Mattia and Merker, 2008). All these are to be considered since, theoretically, living microorganisms by nature may produce the following side effects in susceptible individuals: infection, deleterious metabolic activities, excessive immune stimulation, gene transfer, and antibiotic production (Salminen *et al.*, 1998; Marteau and Shanahan, 2003). Several independent scientific reviews on the safety of lactobacilli have been compiled by qualified experts, which address these safety considerations and present evidence in support of the general safety and history of use of microorganisms belonging to this group of microorganisms (Adams and Marteau, 1995; Salminen *et al.*, 1998; Adams, 1999; Borriello *et al.*, 2003; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2006, 2008; Snydman, 2008). These safety considerations are discussed in detail below.

D.1 Infection

As mentioned, cases of infection due to lactobacilli, including *L. plantarum*, are rare and have occurred exclusively in immunocompromised patients or in patients with severe underlying illnesses, predisposing them to infection (Adams and Marteau, 1995; Salminen *et al.*, 1998;

Adams, 1999; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2006, 2008). In most cases of infection due to lactobacilli, the source of the organism appears to have been the patient's own microflora acting as opportunistic pathogens, and the infections have mainly consisted of endocarditis or bacteremia (Adams and Marteau, 1995; Salminen *et al.*, 1998; Adams, 1999; Borriello *et al.*, 2003; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2006, 2008). Very few cases of infection in immunocompromised patients have been associated with the consumption of foods containing lactobacilli (*e.g.*, fermented foods or probiotic preparations) (Adams and Marteau, 1995; Adams, 1999; Borriello *et al.*, 2003; Bernardeau *et al.*, 2006, 2008; Snydman, 2008). Infections in these latter cases may have been due to contamination of central venous catheters or sintestinal feeding tubes (Bernardeau *et al.*, 2008; Snydman, 2008). Moreover, it has been reported that increased consumption of food products containing lactobacilli over the years has not led to increases in infections due to lactobacilli (Saxelin *et al.*, 1996; Salminen *et al.*, 2002; Bernardeau *et al.*, 2006; Sullivan and Nord, 2006). Based on the findings of the 12-week human study conducted on the HK-LP ingredient (Hirose *et al.*, 2006) and other human studies conducted using live preparations of *L. plantarum* (see Section G), there is no evidence indicating that ingestion of HK-LP would result in infection. The human studies conducted using live preparations of *L. plantarum* further demonstrate the non-pathogenicity of this bacterial species in humans.

D.2 Metabolic Activities

Metabolic activities that are intrinsic to lactobacilli and that theoretically may have harmful effects at high activity levels include the production of biogenic amines, D-lactate, and bacteriocins, platelet aggregating activity, adhesion to the gut mucosa, bile salt deconjugase and dehydroxylase activity, and other enzymatic activities (azoreductase, nitroreductase, β-glucuronidase, glycosidase) (Salminen *et al.*, 1998; Marteau and Shanahan, 2003; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2008); however, these metabolic activities occur naturally due to the activity of endogenous intestinal microflora, and no clinical diseases due to deleterious metabolic effects of probiotic lactobacilli appear to have been reported (Borriello *et al.*, 2003; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2006; Snydman, 2008). In the series of studies conducted by Goossens *et al.* (2003, 2005, 2006) (see Section E), consumption of *L. plantarum* at a daily dose of 10^{11} CFU/person/day did not alter fecal β–glucosidase or β-glucuronidase activity, endotoxin concentration, short-chain fatty acid concentrations, or pH in healthy humans. Furthermore, owing to the fact that HK-LP is non-viable, and therefore, unable to undergo any metabolic functions, the metabolic activities attributed to lactobacilli are likely to be irrelevant. Indeed, upon analysis of 3 non-consecutive lots of the HK-LP ingredient as discussed in Section C, it was confirmed that the biogenic amines histamine and tyramine are not present at detectable levels in the final ingredient as assessed using HPLC analysis. Therefore, the HK-LP ingredient is expected to be free of metabolic activity.

D.3 Immunological Effects

Immunomodulation mediated by cytokines also is a potential side effect of lactobacilli administration (Salminen *et al.*, 1998; Marteau and Shanahan, 2003). However, adverse events of an immunological nature as a result of consumption of food products containing lactobacilli have not been reported in humans (Salminen *et al.*, 1998; Marteau and Shanahan, 2003). Instead, Hirose *et al.* (2006) have demonstrated no adverse effects on acquired or innate immunity in humans following daily ingestion of 50 mg of the HK-LP ingredient over a 12-week period. The equivalent daily dose of HK-LP is 10 mg per day or $1.2x10^{10}$ HK-LP cells per day.

D.4 Antibiotic Resistance Gene Transfer

Lactobacilli, like other bacteria, harbor intrinsic and acquired genes encoding resistance to select antibiotics. For example, certain strains of *L. plantarum* carry resistance to chloramphenicol, vancomycin, or tetracycline (Salminen *et al.*, 1998; Bernardeau *et al.*, 2008; Snydman, 2008). As lactobacilli are sensitive to other common antibiotics, no safety concern is raised. However, a real concern is raised when the bacteria possess antibiotic resistance genes encoded in plasmids. Such genes are transferable and may be transferred to pathogenic bacteria within the gastrointestinal tract (Mathur and Singh, 2005). In most instances, however, the antibiotic resistance gene is intrinsically located within the chromosome of lactobacilli, and therefore, not transferable (Borriello *et al.*, 2003; Marteau and Shanahan, 2003; Snydman, 2008).

D.5 Bacteriocin Production

Bacteriocins are bacterially-produced, small, heat-stable peptides that are active against other bacteria. Such antibacterial substances are naturally produced by many bacterial species, including *L. plantarum* and other food-grade lactic acid bacteria (Eijsink *et al.*, 2002; Cotter *et al.*, 2005; De Vuyst and Leroy, 2007). Although a desirable property for the purpose of preventing contamination of and growth of pathogenic bacteria in food, there exists a concern for the potential development of resistance to bacteriocins among target species. In addition, microorganisms used in food should not produce antibiotic compounds that have similar mechanisms of action to clinically-important antibiotics, particularly if they are readily transferable. HWFC have measured the antibiotic activity of three non-consecutive lots of the HK-LP ingredient (Lot Nos. 060621, 080524, and 081114) by the method described by the Joint FAO/WHO Expert committee on Food Additives in the Combined Compendium of Food Additive Specifications (JECFA, 2006). The final ingredient did not exhibit antibiotic activity, and therefore, is not a concern in this regard and would not have implications in the development of resistance to bacteriocins (see Appendix C).

E. Fate of HK-LP in the Gastrointestinal Tract

In normal healthy individuals, the intestinal mucosal barrier is impermeable to bacteria. The escape of bacteria from the gastrointestinal tract and across the intestinal mucosal barrier is referred to as bacterial translocation and is an unusual and undesirable effect occurring in individuals with compromised gastrointestinal tracts (Ishibashi and Yamazaki, 2001; Lichtman, 2001; Liong, 2008). Specifically, the definition of 'bacterial translocation' is "the passage of *viable* resident bacteria from the gastrointestinal tract to normally sterile tissues" (MacFie, 2004). In cases of bacterial translocation, bacteria are transported to the mesenteric lymph nodes, and subsequently, to the liver, spleen, and general circulation, with the potential to result in bacteremia, sepsis, and multiple organ failure. However, considering that bacterial translocation refers to *viable* bacteria, the phenomenon and the adverse outcomes associated with it do not apply to HK-LP, which is non-viable. Furthermore, given that HK-LP retains the form of intact bacteria, the potential for HK-LP to enter organs and the systemic circulation from the gastrointestinal tract is likely to be non-existent in normal healthy individuals. Instead, the fate of HK-LP following ingestion is expected to be equivalent to that of live food-grade bacteria that is consumed. In other words, HK-LP is expected to transit through the gastrointestinal tract and be excreted in the feces. Moreover, the animal studies described in Section F provide evidence that live *L. plantarum* from orally-administered preparations do not harbor potential for translocation (*i.e.*, absorption of *L. plantarum* from the gastrointestinal tract does not occur) (Pavan *et al.*, 2003; Daniel *et al.*, 2006). Human studies described in Section G also provide evidence for the safety of *L. plantarum* as it pertains to bacterial translocation.

F. Toxicological Studies

F.1 Acute Toxicity Studies

HK-LP has been reported to have low acute oral toxicity in experimental mice. An HK-LP ingredient, consisting of 50% HK-LP and 50% dextrin and referred to as 'LP20 prototype', was assessed in an acute oral toxicity study (Hirose *et al.*, 2009). The study was performed according to Japan's Ministry of Health, Labor, and Welfare Revised Guidelines for Single and Repeated Dose Toxicity Studies (Notification No. 88 of the Pharmaceuticals and Cosmetics Division, PAD dated August 10, 1993). Groups of 5 male and 5 female specific pathogen-free Slc:ICR mice were administered a single gavage dose of 2,000 mg/kg body weight of LP20 prototype dissolved in water. The animals were observed for a period of 14 days after administration for toxicological symptoms and mortality. Body weights were recorded on Days 7 and 14 and macroscopic findings recorded at necropsy. No mortality and no signs of toxicity were observed in any of the animals throughout the observation period. One female exhibited reduced body weight gain at 1 week after administration of LP20 prototype compared to the other females; however, the body weight of this animal increased steadily thereafter. All other animals displayed normal body weight changes throughout the observation period and final

body weights were similar within the female and male groups. Upon necropsy, ovarian cysts were observed in 3 of the 5 females, but were considered to be a spontaneous lesion and not related to acute treatment with LP20 prototype as cysts are unlikely to have developed over the 14-day timeframe of this study. Furthermore, ovarian cysts are commonly found in ageing mice of many strains and also have been reported to occur earlier on in the lifespan of mice (Montgomery and Alison, 1987; Maekawa *et al.*, 1996), although, their presence in younger mice has not been well studied since few historical control data are available from which to make comparisons (*i.e.*, ovarian histopathology data on young mice are lacking). Based on the results of this study, the authors reported the acute oral LD_{50} value for LP20 prototype, consisting of 50% HK-LP and 50% dextrin, to be greater than 2,000 mg/kg body weight in both male and female mice. Therefore, the acute oral LD_{50} value for HK-LP in mice was considered to be greater than 1,000 mg/kg body weight.

F.2 Short-Term Studies

Traditional repeat dose toxicological studies have not been performed on the HK-LP ingredient; however, several studies involving short-term repeat administration of live preparations of *L. plantarum* were identified in the publicly available scientific literature. Three of the identified studies included abbreviated safety assessments and other studies were aimed at elucidating the effect of *L. plantarum* consumption in improving the condition of experimental animals in diseased or compromised states. The results of the abbreviated safety assessments are summarized in the following paragraphs. Together, this data demonstrated that short-term oral administration of live preparations of *L. plantarum* at doses of up to 525 mg/kg body weight/day (providing up to 10^{10} CFU/day) did not produce adverse effects on standard parameters of toxicological testing. The results of these studies further indicate that *L. plantarum* does not harbor potential for bacterial translocation, including in cases of increased intestinal permeability.

L. plantarum NCIMB8826 (isolated from human saliva) was assessed for potential toxicity in both healthy mice and mice with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, a mouse model for inflammatory bowel disease (Pavan *et al.*, 2003). Groups of 5 to 9 male BALB/c mice received a daily dose of 10^9 CFU of either *L. plantarum* NCIMB8826 or *Lactococcus lactis* MG1363 by oral gavage for 4 consecutive days. The amount of *L. plantarum* administered was approximately equivalent to 1.1 mg on a dry weight basis. Assuming a body weight of 0.020 kg for adult mice (U.S. FDA, 1993) and using the calculation by Passos *et al.* (1994) that $2.5x10^{11}$ of viable *L. plantarum* cells weigh 0.264 g on a dry weight basis, the mice were estimated to have been administered *L. plantarum* at a dose of 55 mg/kg body weight/day. Control mice received buffer (vehicle control). Healthy groups of mice were euthanized on the day following the last day of administration (Day 5), while the remaining groups of mice were treated with TNBS or ethanol (TNBS control) on Day 5 and euthanized on Day 7. Body weight was recorded daily and mice were scored for activity. At necropsy, macroscopic and

histological examinations of the colon and small intestine were conducted and mesenteric lymph nodes and spleen were removed and assessed for bacterial content. In healthy mice, no variations in mouse activity or body weight were observed following 4 days of bacteria administration compared to controls. Also, no signs of macroscopic or histological inflammation were detected in the ileum or colon. Furthermore, neutrophil recruitment to tissues of the colon and small intestine was not observed, and mesenteric lymph nodes and spleen cultures were negative for *L. plantarum* NCIMB8826 and *Lactococcus lactis* MG1363. All groups of healthy mice, including those administered *L. plantarum* NCIMB8826, displayed low numbers of endogenous microflora in mesenteric lymph nodes and spleens, whereas in TNBS-treated mice, the endogenous microflora was identified in amounts 10^7-fold greater. Despite an increase in intestinal permeability, as evidenced by translocation of the endogenous microflora, the administered *L. plantarum* or *Lactococcus lactis* strains were not detected in the mesenteric lymph nodes or spleens of TNBS-treated mice. The authors concluded that "feeding live *L. plantarum* induced no detrimental effects and no abnormal translocation of the administered bacteria".

A safety investigation of a similar nature was conducted by Daniel *et al.* (2006), also with the use of a mouse colitis model. Groups of 10, 7-week-old female BALB/c mice were intragastrically administered *L. plantarum* Lp-115 (isolated from plant material), *Lactobacillus salivarius* Ls-33, *L. acidophilus* NCFM, or *Lactobacillus paracasei* YS8866441 at a dose of 10^{10} CFU/day for 5 consecutive days. The amount of *L. plantarum* administered was approximately equivalent to 10.5 mg on a dry weight basis. Using the same calculation as above, the mice were estimated to have been administered *L. plantarum* at a dose of 525 mg/kg body weight/day. Control mice received vehicle alone. As above, colitis was induced on Day 5 with TNBS. Both healthy and TNBS-treated mice were euthanized on Day 7. Mice were weighed prior to TNBS administration and at study end. Mice were observed for mortality and also were scored for activity as above. Colonic damage, including colonic macroscopic inflammation scores, was assessed 48 hours following treatment with TNBS. Mesenteric lymph nodes, spleen, liver, and kidneys also were removed at necropsy and examined for bacterial content. In healthy mice, no adverse effects were observed with regards to activity, body weight, colonic inflammation, or neutrophil recruitment in colonic tissues following administration of bacteria compared to controls. Additionally, mesenteric lymph nodes, spleen, liver, and kidneys were negative for the administered bacteria in all healthy and most TNBS-treated mice. In one TNBS-treated mouse presenting with 'very strong colitis', *L. plantarum* Lp-155 was detected at low levels in the liver and kidneys, but was without any adverse effects. Again, all groups of healthy mice, including that administered *L. plantarum* Lp-115, displayed low numbers of endogenous microflora in the organs examined, whereas TNBS-treated mice displayed much greater levels of translocation of the endogenous microflora. The authors' conclusions were consistent with those of Pavan *et al.* (2003), that orally administered *L. plantarum* Lp-155, at a dose of 10^{10} CFU/day, did not induce adverse effects or abnormal translocation of the bacteria administered even in mice with increased intestinal permeability.

In the third study identified, 5- to 6-week-old male ICR mice were fed a high-cholesterol diet containing 10% w/v skim milk and 10% w/v cream for 14 days (Nguyen *et al.*, 2007). Mice (6/group) were subsequently orally administered (method of administration not specified) a daily dose of 10^7 CFU of *L. plantarum* pH04 (isolated from fecal samples from human newborn infants) or no bacteria for an additional 14 days. The amount of *L. plantarum* administered was approximately equivalent to 0.01 mg on a dry weight basis. As calculated above, the mice were estimated to have been administered *L. plantarum* at a dose of 0.5 mg/kg body weight/day. There is no indication that a vehicle was administered to control animals. The activity, behavior, and general health of mice were monitored daily. Food and water intake and body weights were measured weekly. Fecal bacteria counts were determined before and after the 14-day *L. plantarum* pH04 administration period on a per gram fecal wet weight basis. Upon necropsy following the 14-day administration period, blood samples were collected and serum was analyzed for total cholesterol and triglycerides. In addition, mesenteric lymph nodes, spleen, and liver were excised and examined for bacterial translocation. Blood samples also were examined for bacterial translocation. No significant differences were observed among groups for food intake, body weight, visceral weight indices for lymph nodes, spleen, and liver, or behavior. Serum total cholesterol and triglycerides were significantly reduced in the *L. plantarum* pH04 group compared to the no bacteria control group. In addition, mice administered *L. plantarum* exhibited a significant increase in fecal lactic acid bacteria content compared to mice in the control group. Furthermore, the occurrence of bacterial translocation was similar among groups, with one sample of each tissue (mesenteric lymph nodes, spleen, and liver) from each group being positive for translocation. With respect to the metabolic activity of *L. plantarum* pH04, the investigators noted that this strain of *L. plantarum* displayed bile salt hydrolase activity *in vitro* induced by conjugated bile salts, but not by deconjugated bile salts. *L. plantarum* pH04 also failed to produce the enzyme β-glucuronidase.

L. plantarum 299v was administered to different age groups of Sprague-Dawley rats on Post-Natal Days (PND) 3 to 10, 7 to 14, or 14 to 21 (Fåk *et al.*, 2008). Approximately 8 to 11 rats per group were used, each of which was paired to a separate age-matched control group. The animals were administered the bacteria at a dose of 3.0×10^9 CFU/kg body weight/day, equivalent to a dose of 3.4 mg/kg body weight/day. Control animals were gavaged with saline. Following the exposure period, the rats were sacrificed and a weight and content analysis of the small intestine, the pancreas, blood plasma, and cecum bacteria were conducted. Administration of *L. plantarum* 299v did not result in any adverse effects (*e.g.*, diarrhea or behavioral changes) or body weight gain differences in any of the animals tested. The number of cecal lactobacilli increased significantly in the 2 youngest treatment groups (*i.e.*, mice administered *L. plantarum* throughout PND 3 to 10 or 7 to 14) compared to controls (10-fold and 2-fold increase, respectively), while the number of *Enterobacteriaceae* did not differ significantly between groups. No significant compound-related effects were observed on relative organ weights for the liver, stomach, small intestine, cecum, spleen, and thymus, or on the pH of the stomach contents compared to controls. An exception was a significant, yet slight, reduction in

the weight of the distal portion of the small intestine in the oldest *L. plantarum* 299v group (*i.e.*, mice administered *L. plantarum* throughout PND 14 to 21). A significant decrease in the absorption of marker molecules for intestinal barrier function (bovine immunoglobulin G and bovine serum albumin) was observed in the youngest *L. plantarum* 299v group based on significantly decreased plasma levels compared to controls. This result was an indication of increased macromolecular barrier function in the small intestine. Administration of *L. plantarum* 299v resulted in a significant decrease in the weight of the pancreas and adrenals and pancreatic protein content in the rats dosed from PND 7 to 14 compared to controls. A reduction in pancreas weight also was noted in the other 2 groups, but these did not reach statistical significance. The findings observed in the pancreas were suggested by the authors to be a result of an establishment of lactobacilli flora with more efficient digestive capabilities compared to that of control animals who may have increased the production of pancreatic digestive enzymes. The observations detected in the adrenals were suggested to be a result of a reduction in stress as discussed by the authors. The results of this study support the safety of orally-administered *L plantarum* 299v in immature rats in that *L. plantarum* 299v administration did not adversely impact intestinal barrier function in either neonatal or postnatal rats.

Several other studies involving repeat oral administration of various strains of *L. plantarum* to experimental animals were identified in the publicly available literature; however, such studies were aimed at elucidating the effect of *L. plantarum* consumption in improving the condition of experimental animals in diseased or compromised states and did not specifically address the safety of orally administered *L. plantarum* in and of itself. Nonetheless, the results of these studies demonstrated that orally administered *L. plantarum* did not negatively affect the frequency of bacterial translocation (by potentially pathogenic bacteria or *Lactobacillus* species) in experimental mice and rats and did not have any adverse impact on the condition in question (Mao *et al.*, 1996; Kasravi *et al.*, 1997; Kennedy *et al.*, 2000; Liu *et al.*, 2001; Wang *et al.*, 2001; Schultz *et al.*, 2002; von Bültzingslöwen *et al.*, 2003; Dieleman *et al.*, 2003; Osman *et al.*, 2004, 2007, 2008; White *et al.*, 2006; Håkansson *et al.*, 2006; Han *et al.*, 2006; Mangell *et al.*, 2006; Gross *et al.*, 2008).

F.3 Mutagenicity and Genotoxicity Studies

The HK-LP ingredient has been shown to be non-mutagenic and non-genotoxic in both *in vitro* and *in vivo* validated systems (Hirose *et al.*, 2009). The HK-LP ingredient was shown to be without mutagenic activity in the bacterial reverse mutation assay (Ames test) using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2*uvrA*. The ingredient was non-mutagenic and also was not cytotoxic at concentrations of up to 5,000 µg/plate in either the presence or absence of S9 metabolic activation. The HK-LP ingredient was further demonstrated to be without clastogenicity in a chromosome aberration assay conducted in a Chinese hamster lung (CHL) fibroblast cell line. Specifically, the ingredient was negative for inducing numerically or structurally abnormal chromosomes at

concentrations of up to 5,000 µg/mL with or without metabolic activation, and also did not produce cytotoxicity. *In vivo*, the HK-LP ingredient was shown to be non-genotoxic in the mouse micronucleus assay. In this test, groups of 5 male specific pathogen free Crlj:CD1 mice were orally administered 2 consecutive daily doses of 500, 1,000, or 2,000 mg/kg body weight of the ingredient by gavage. No mortalities and no changes in body weight occurred at any dose level and no significant differences in the incidence of micronucleated polychromiatic erythrocytes or polychromatic erythrocytes were observed compared to the negative control group. Therefore, the HK-LP ingredient is not considered to have mutagenic or genotoxic potential.

F.4 Other Studies

One study was identified in the publicly available literature in which HK-LP was tested for anti-tumor properties (Kim *et al.*, 2002). Six-week-old male BALB/c mice were randomized into 1 of 7 groups (6/group) to receive whole HK-LP cells (100 mg/kg body weight/day), cytoplasmic fractions of *L. plantarum* (100 or 200 mg/kg body weight/day), or vehicle control by oral gavage for 7 days prior to or after tumor cell inoculation [intraperitoneal injection with F9 teratocarcinoma cells (10^6 cells/animal)]. Compared to the control group, survival rates were not adversely affected by administration of *L. plantarum* as either a heat-killed whole cell or a cytoplasmic preparation. Body weight was observed not to be significantly different between groups throughout the course of the study. Although no other specific safety parameters were measured, the authors reported that toxicity and marked side effects were not observed in animals receiving the cytoplasmic fractions. Whether toxicity or side effects occurred in animals receiving the whole cell preparation was not reported.

Two other studies, specifically on HK-LP, were identified in the publicly available literature that involved intraperitoneal administration of HK-LP to mice (50 to 500 µg of HK-LP suspended in saline and injected intraperitoneally for 4 to 7 days) (Murosaki *et al.*, 1998, 2000). Although body weights and survival rates were not adversely affected by HK-LP administration, these studies impart little value in the safety evaluation of HK-LP as a food ingredient given that HK-LP was administered intraperitoneally and there was no specific reference to the measurement of any safety parameters.

G. Studies in Humans

The safety of the HK-LP ingredient has been evaluated in healthy human volunteers, the results of which have been published in the scientific literature and which are detailed below. In addition, numerous human studies were identified in the scientific literature in which commercially available live preparations of *L. plantarum* were provided orally as probiotic supplementations to various human subpopulations, including healthy volunteers, patient population groups, and infants. The results of these studies support the safety of *L. plantarum* for human consumption as described below. Traditional safety evaluations have not been

performed on live preparations of *L. plantarum* in humans, however, this may be due to the general consensus that lactobacilli are considered safe for human consumption due to their long history of use in foods, and therefore, traditional safety studies have likely been considered unnecessary.

G.1 HK-LP

The safety of the HK-LP ingredient was assessed within a double-blind, randomized, placebo-controlled study conducted by Hirose *et al.* (2006). Sixty (60) healthy men and women (18 men and 18 women aged 40 to 64 years and 12 men and 12 women aged >64 years) received 1 capsule providing either 50 mg of the HK-LP ingredient or dextrin (placebo) daily for a period of 12 weeks. Subjects were estimated to have consumed approximately $1.2x10^{10}$ HK-LP cells daily. The safety assessment included anthropometric measurements, biochemical examinations of blood, hematological assessments, and urine tests, which were performed at baseline and at Week 12. A health-related quality of life questionnaire also was completed by subjects every 4 weeks. Although the results of the safety assessment were not shown, the authors reported that no significant changes were detected in the parameters measured between the HK-LP and placebo groups at Week 12. An exception was a significant increase in lymphocyte count in the HK-LP group ($p = 0.03$) compared to the placebo group; however, the lymphocyte count value of the HK-LP group was reported to remain within the range of reference values. Additionally, the quality of life score was not adversely affected in the HK-LP group compared to baseline and control group scores. Several adverse events were reported, including 3 in the placebo group and 6 in the HK-LP group. In the control group, upper respiratory tract infections (2) and knee arthralgia (1) were reported. In the HK-LP group, chronic ulcerative colitis (1), herniated disk (1), Menier's syndrome (1), dizziness (1), herpes labialis (1), and acute urticaria (1) were reported. The subject with chronic ulcerative colitis and the subject with the herniated disk both dropped out of the study. The authors reported that none of the adverse events were attributed to HK-LP supplementation.

Furthermore, the HK-LP ingredient has been consumed as a supplement, which is classified as an 'encapsulated food', providing 52 mg of the ingredient since 2005 in Japan, and, since 2008, as an ingredient of a Japanese granular-powder food product, which contains 50 mg/serving of the HK-LP ingredient. Such intake over the past 4 years of the supplement has not led to reports of adverse reactions. Five inquiries into the possible association of this supplement with constipation have been reported; however, no causal relationship could be identified.

G.2 Live Preparations of *Lactobacillus plantarum* in Adults

Numerous human studies were identified in which commercially-available preparations containing live *L. plantarum* strain 299v were provided orally as probiotic supplementations to healthy subjects as well as in various patient population groups. All studies were conducted with the use of preparations containing only *L. plantarum* and not in combination with other

microorganisms. The findings from these studies are summarized below and the details of each study are presented in Table G.2-1.

Studies conducted in healthy volunteers were double-blinded, randomized, and placebo-controlled and were conducted in both males and females (Bukowska *et al.*, 1998; Johansson *et al.*, 1998; Naruszewicz *et al.*, 2002; Goossens *et al.*, 2003, 2005, 2006). In these studies, subjects consumed *L. plantarum* 299v, delivered in a fermented oatmeal, fruit drink, at daily doses of 10^{10} to $2x10^{11}$ CFU per person for periods of 2 to 6 weeks with good tolerability. Many of the endpoints measured that may be considered relevant to safety were related to colonic health, including colonic microbial fermentation [*i.e.*, the production of fecal short-chain fatty acids (acetic acid, propionic acid, butyric acid) and lactic acid], fecal pH, and fecal microflora counts. No untoward effects were observed with regards to these endpoints. As expected, consumption of live *L. plantarum* 299v resulted in increased fecal counts for the ingested strain and for the general group of lactic acid bacteria (lactobacilli and bifidobacteria). However, fecal counts for all other microorganisms, including those for potentially pathogenic microorganisms, remained unchanged. Stool frequency and consistency also were unaffected in healthy population groups. In addition, in one study conducted in smokers and moderately hypercholesteremic, but otherwise healthy men, parameters related to cardiovascular health, including systolic and diastolic blood pressure, blood lipid levels, and plasma fibrinogen levels, as well as plasma glucose and insulin levels, were unchanged or not adversely affected by ingestion of live *L. plantarum* 299v compared to placebo (Bukowska *et al.*, 1998; Naruszewicz *et al.*, 2002). Moreover, no adverse events or side effects attributable to the bacterial preparation occurred in any of the studies.

Several double-blinded, randomized, and placebo-controlled studies also have been conducted in patients diagnosed with irritable bowel syndrome (IBS), a gastrointestinal disorder (Nobaek *et al.*, 2000; Nieldzielin *et al.*, 2001; Sen *et al.*, 2002). All studies were 4 weeks in duration in which patients were provided with *L. plantarum* 299v at a daily dose of $6.25x10^9$ or $2x10^{10}$ CFU per person in a fermented oatmeal, fruit drink. The drinks were well tolerated and no adverse events or side effects attributable to the bacterial preparation occurred in any of the studies. Additionally, ingestion of live *L. plantarum* 299v by IBS patients did not exacerbate symptoms of IBS, such as abdominal pain, irregular bowel movements, and flatulence.

Other studies included those conducted in patients with *C. difficile*-associated diarrhea (Wullt *et al.*, 2003, 2007), as well as those conducted in hospitalized patient population groups, which consisted of liver transplant recipients (Rayes *et al.*, 2002), patients undergoing elective major abdominal surgery (McNaught *et al.*, 2002), critically-ill patients admitted to the intensive care unit (McNaught *et al.*, 2005; Klarin *et al.*, 2008), and patients with acute pancreatitis (Qin *et al.*, 2008). Subjects were provided with *L. plantarum* at a daily dose of $5x10^7$ to $8x10^{10}$ CFU per person delivered in a fermented oatmeal, fruit drink or in an enterally fed formula over short-term periods of 7 to 12 days. The objective of these studies was to determine the effects of live

preparations of *L. plantarum* on bacterial translocation and parameters related to inflammation and infection, including systemic inflammatory response syndrome, catheter-related septic complications, positive blood culture, lymphocyte counts, and serum biomarkers of inflammation [*e.g.*, C-reactive protein (CRP), tumor necrosis factor-α, and interleukin (IL) levels]. The results of these studies demonstrated that the ingestion of live *L. plantarum* failed to increase intestinal permeability or incidences of bacterial translocation and septic-complications in individuals susceptible to infection (McNaught *et al.*, 2002, 2005; Wullt *et al.*, 2003, 2007; Klarin *et al.*, 2008; Qin *et al.*, 2008).

The above data demonstrate that the consumption of *L. plantarum*, at doses relevant to the intended the use levels of the HK-LP ingredient, are not associated adverse effect in humans.

Table G.2-1 Oral Human Studies Conducted on LP20, Heat-Killed *Lactobacillus plantarum* (HK-LP), and Live *L. plantarum*

Study Population	Study Design	Dose	Duration	Observations[a]	Reference
Heat-Killed *Lactobacillus plantarum* L-137 (HK-LP)					
60 Healthy subjects (18M, 18F, aged 40 to 64 y; 12M, 12F, aged >64 y)	Double-blind, randomized, placebo-controlled	Capsules containing 0 (placebo) or **50 mg LP20**/day, providing **~1.2x10^{10} HK-LP cells/day**	12 weeks	No adverse events were attributed to LP20 supplementation. ↑ Lymphocyte count in LP20 group was within reference range. NSD in other anthropometric measurements, biochemical examinations of blood, hematological assessments, and urine tests. Quality of life scores not adversely affected by treatment.	Hirose *et al.*, 2006
***Lactobacillus plantarum* 299v (LP 299v)**					
Healthy Subjects					
29 Healthy subjects[1] (9M, mean age 34 y; 20F, mean age 26 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **2x10^{11} CFU/day** (delivered as a 200-mL fermented oatmeal drink containing 10^{9} CFU/mL of LP 299v) taken with or without 40 mg/day of pantoprazole	2 weeks	No side effects were reported by subjects. NSD in stool frequency or consistency. NSD in fecal short-chain fatty acid concentrations, pH, and counts for microorganisms. Gastric acid inhibition with pantoprazole had no effect on the parameters assessed.	Goossens *et al.*, 2005
29 Healthy subjects (16M, 13F; mean age 56.3 to 57.5 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **2x10^{11} CFU/day** (delivered as a 200-mL fermented oatmeal drink with 10^{9} CFU/mL of LP 299v)	2 weeks	No side effects were reported by subjects. ↑ Number of total anaerobic bacteria in rectal biopsy samples of LP group, but NSD were detected in rectum or colon for all other bacterial groups.	Goossens *et al.*, 2006

[1] A total of 32 subjects enrolled in the study; however, 3 subjects were excluded from the study; one subject did not take the pantoprazole/placebo for personal reasons and 2 subjects took antibiotics at some point during the study.

Table G.2-1 Oral Human Studies Conducted on LP20, Heat-Killed *Lactobacillus plantarum* (HK-LP), and Live *L. plantarum*

Study Population	Study Design	Dose	Duration	Observations[a]	Reference
48 Healthy subjects (11M, 37F; mean age of 36 to 37 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **$2x10^{10}$ CFU/day** (delivered as a 400-mL fermented oatmeal, fruit drink containing $5x10^{7}$ CFU/mL of LP 299v)	3 weeks	Drinks were well tolerated. NSD in incidences of adverse events (transient symptoms of nausea, abdominal discomfort, and flu-like symptoms). NSD in stool frequency or consistency or in difficulty with defecation. ↑ Stool volume and ↓ flatulence in LP 299v group. NSD in fecal pH.	Johansson *et al.*, 1998
20 Healthy subjects[2] (9M, mean age 34 y; 11F, mean age 31 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **$2x10^{11}$ CFU/day** (delivered as a 200-mL fermented oatmeal drink containing 10^{9} CFU/mL of LP 299v)	4 weeks	NSD in stool frequency or consistency. NSD in fecal β–glucosidase or β-glucuronidase activity, endotoxin concentration, short-chain fatty acid concentrations, or pH. ↑ Lactobacilli counts in LP group, but NSD detected in counts for all other microorganisms.	Goossens *et al.*, 2003
36 Current but otherwise healthy smokers (18M, 18F, aged 35 to 35 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **$2x10^{10}$ CFU/day** (delivered as a 400-mL fruit drink containing $5x10^{7}$ CFU/mL of LP 299v)	6 weeks	Drinks were well tolerated and no adverse events were reported. NSD in systolic and diastolic blood pressure or in plasma triacylglycerol, total cholesterol, LDL cholesterol, HDL cholesterol, lipoprotein(a), homocysteine, glucose, or insulin levels. ↓ Plasma leptin and fibrinogen levels in LP group.	Naruszewicz *et al.*, 2002
30 Moderately hypercholesteremic but otherwise healthy men (mean age of 42 to 43 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **10^{10} CFU/day** (delivered as a 200-mL fruit drink containing $5x10^{7}$ CFU/mL of LP 299v)	6 weeks	NSD in serum cholesterol, serum LDL-cholesterol, plasma fibrinogen, serum triglyceride, serum HDL-cholesterol, or plasma glucose levels.	Bukowska *et al.*, 1998

[2] A total of 22 subjects enrolled in the study, however, 2 subjects in the placebo group were excluded; one subject was unable to collect 80% of the fecal samples and one subject did not tolerate the fermented oatmeal drink.

Table G.2-1 Oral Human Studies Conducted on LP20, Heat-Killed *Lactobacillus plantarum* (HK-LP), and Live *L. plantarum*

Study Population	Study Design	Dose	Duration	Observations[a]	Reference
Patients with Irritable Bowel Syndrome (IBS)					
52 IBS patients (16M, 36F; aged 21 to 78 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **$2x10^{10}$ CFU/day** (delivered as a 400-mL fermented oatmeal, fruit drink containing $5x10^7$ CFU/mL of LP 299v)	4 weeks	Drinks were well tolerated by all patients and no adverse events were reported. NSD in abdominal pain and stool frequency and consistency. ↓ Flatulence in LP group.	Nobaek *et al.*, 2000
40 IBS patients (8M, 32F; mean age of 42 to 45 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **$2x10^{10}$ CFU/day** (delivered as a 400-mL fermented oatmeal, fruit drink containing $5x10^7$ CFU/mL of LP 299v)	4 weeks	No compound-related side effects were observed. NSD in stool frequency or consistency. ↓ Abdominal pain, flatulence, and overall symptom scores in LP group.	Nieldzielin *et al.*, 2001
12 IBS patients (1M, 11F; aged 23 to 61 y)	Double-blind, randomized, placebo-controlled	**$6.25x10^9$ CFU/day** (delivered as a 125-mL fermented oatmeal, fruit drink containing $5x10^7$ CFU/mL of LP 299v)	4 weeks	NSD in daily symptom scores for abdominal pain frequency and severity, abdominal distension, stool frequency and urgency, and flatulence. NSD in total and individual (hydrogen and methane) gas production and maximum rates of gas production.	Sen *et al.*, 2002
Patients with *Clostridium difficile*-Associated Diarrhea					
21 patients with recurrent *C. difficile*-associated diarrhea (1M, 20F; mean age of 63 to 65 y)	Double-blind, randomized, placebo-controlled	0 (placebo) or **$5x10^7$ CFU/day** (delivered as a fermented oatmeal, fruit drink containing $5x10^7$ CFU of LP 299v)	10 days during antibiotic treatment plus an additional 28 days	No side effects were reported by subjects. NSD in recurrence of clinical symptoms of *C. difficile*-associated diarrhea or in bacteriological recurrence or persistence. ↓ Fecal butyrate concentration in placebo group (Days 11 to 13 only) compared to LP group; otherwise NSD in fecal short-chain fatty acid and organic acid content.	Wullt *et al.*, 2003, 2007

Table G.2-1 Oral Human Studies Conducted on LP20, Heat-Killed *Lactobacillus plantarum* (HK-LP), and Live *L. plantarum*

Study Population	Study Design	Dose	Duration	Observations[a]	Reference
Hospitalized Patient Population Groups					
95 Liver transplant recipients (49M, 46F, mean age of 47 to 50 y)	Double-blind, randomized, placebo-controlled	0 (placebo), **10^9 CFU HK-LP 299v, or 10^9 CFU live LP 299v/day** (delivered by enteral feeding, heat-killed culture served as placebo; no bacteria preparation served as an additional control)	12 days	All formulas were well tolerated, and abdominal side effects (distension, cramps, and diarrhea) were reported in all groups at similar frequencies. ↓ Incidence of postoperative infections in live LP group and NSD between HK-LP and no bacteria groups. ↑ BUN levels in no bacteria group compared to the live LP group. NSD in other clinical chemistry, hematology, and immunological parameters.	Rayes *et al.*, 2002
103 critically-ill patients in ICU (58M, 45F; aged 28 to 90 y)	Randomized	0 (control) or **$2.5x10^{10}$ CFU/day** (delivered as a 500-mL fermented oatmeal drink containing $5x10^7$ CFU/mL of LP 299v; non-placebo control group received no intervention)	Median intake = 9 days	NSD in gastric colonization, endotoxin exposure, septic morbidity, intestinal permeability, and serum CRP levels. ↓ Serum IL-6 levels in LP group (Day 15 only).	McNaught *et al.*, 2005
129 patients undergoing elective major abdominal surgery (75M, 54F; aged 58 to 77 y)	Randomized	0 (control) or **$2.5x10^{10}$ CFU/day** (delivered as a 500-mL fermented oatmeal drink containing $5x10^7$ CFU/mL of LP 299v, non-placebo control group received no intervention)	7 days preoperatively as well as during the postoperative period	NSD in extent of gastric colonization. NSD in the overall prevalence of bacterial translocation to the mesenteric lymph node or small intestine. NSD in serum CRP levels or in the incidence of septic morbidity.	McNaught *et al.*, 2002

0000039

Table G.2-1 Oral Human Studies Conducted on LP20, Heat-Killed *Lactobacillus plantarum* (HK-LP), and Live *L. plantarum*

Study Population	Study Design	Dose	Duration	Observations[a]	Reference
74 patients with acute pancreatitis (23M, 51F; mean age of 54 to 58 y)	Randomized, single-blinded, parallel study	0 (control) or **10^{10} CFU/day** (delivered as 100 mL/day of 10^8 CFU/mL of LP or saline *via nasojejunal tube*)	7 days	No adverse events. NSD in the total number of positive isolates in nasogastric aspirates ↓ Number of patients with aspirates colonized by multiple organisms or potentially pathogenic organisms in LP group NSD in amount of fecal enterobacteria. ↑ Fecal bifidobacteria and lactobacteria and ↓ in enterococci in LP group. ↓ in severity of systemic inflammatory response syndrome, catheter-related septic complications, positive blood culture, lymphocyte cell count, and serum CRP levels in LP group. ↓ Intestinal permeability in LP group (Days 5 & 8).	Qin *et al.*, 2008
44 critically-ill patients in ICU (26M, 18F, aged 18 to 89 y)	Randomized, placebo-controlled	0 (placebo) or **$8x10^{10}$ CFU/day** (delivered by enteral feeding of a fermented oatmeal drink containing $8x10^8$ CFU/mL of LP 299v)	Patients were enterally fed the preparation initially as 6 100-mL doses at 12-hour intervals and thereafter at 50 mL twice/day for the duration of their stay	Both preparations were well tolerated and no adverse impact of the LP preparation was observed. No overall differences in the frequency of bowel movements or stool consistency. ↓ Number of patients with *C. difficile* positive samples and ↑ in the number of patients with LP 299v positive samples in LP group. NSD in frequencies of other positive cultures, serum CRP, tumor necrosis factor-α, IL-1β, IL-6, and IL-10 levels. NSD in intestinal permeability.	Klarin *et al.*, 2008

Abbreviations: *C. difficile* = *Clostridium difficile*; CRP = C-reactive protein; F = female; ICU = intensive care unit; IBS = Irritable Bowel Syndrome; IL = interleukin; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LP = *Lactobacillus plantarum*; M = male; NSD = no significant differences; y = years.
[a] All differences noted are between the *L. plantarum* and control groups and were reported as statistically significantly different; within-group differences (*i.e.*, compared to baseline measurements) are not noted in the table unless otherwise stated.

G.3 Live Preparations of *Lactobacillus plantarum* in Infants and Children

Infants and children represent a special population group whose safety concerns need to be specifically addressed since the majority of safety information is based on the adult population. The major concern in this regard is the potential of bacterial translocation in those with developing and immature gastrointestinal systems. The human neonatal intestinal barrier is indeed permeable to macromolecules, particularly in pre-term infants (Axelsson *et al.*, 1989; Kuitunen *et al.*, 1994). As a result of an immature intestinal barrier, pre-term infants are susceptible to translocation of pathogenic bacteria and to sepsis (Westerbeek *et al.*, 2006; Anand *et al.*, 2007); however, with increasing postnatal age and maturity, the intestinal barrier becomes substantially less permeable (Axelsson *et al.*, 1989; Kuitunen *et al.*, 1994), decreasing the susceptibility to bacterial translocation. In addition, lactobacilli are not pathogenic and exist as beneficial commensal bacteria present as part of the normal intestinal microflora of healthy humans (Molin *et al.*, 1993; Ahrné *et al.*, 1998; Song *et al.*, 2000). *L. plantarum*, specifically, has been isolated from fecal samples from healthy infants (Park *et al.*, 2002; Ahrné *et al.*, 2005; Nguyen *et al.*, 2007) and children (Annuk *et al.*, 2001), demonstrating the occurrence of *L. plantarum* as part of the natural intestinal microflora beginning from as early as the newborn stage. Moreover, a delay in intestinal colonization with beneficial bacteria, such as lactobacilli, may instead increase the susceptibility of pre-term infants to bacterial translocation involving pathogenic bacteria (Van Camp *et al.*, 1994; Westerbeek *et al.*, 2006; Claud and Walker, 2008; Hunter *et al.*, 2008). Therefore, lactobacilli are thought to aid in the prevention of bacterial translocation in cases of pre-term infancy in which the intestinal barrier is not fully developed instead of resulting in detrimental effects.

With respect to the safety of *L. plantarum* intake by infants, 1 randomized, double-blind study was identified in the scientific literature. Healthy newborn infants, all delivered by caesarean section, received daily either a synbiotic preparation containing 10^9 *L. plantarum* cells and 150 mg of fructooligsaccharides dissolved in dextrose saline (7 males and 12 females) or dextrose saline alone (5 males and 7 females) for a duration of 7 days (Panigrahi *et al.*, 2008). Weight at birth, Day 7, and Day 28 was recorded. Safety and tolerance data also were recorded and included vital signs, feeding and stooling patterns, signs of sepsis, and any other medical events considered important. Stool samples were collected at baseline and on Days 3, 7, 14, and 28 for microbiological analysis. The percent change in weight from baseline did not differ significantly between groups on Day 7, but was significantly increased on Day 28 in infants receiving *L. plantarum* compared to those receiving control. On Days 21 and 28, the total number of bacterial species was significantly increased in stool samples from the *L. plantarum* group compared to the control group as a result of an increase in the number of gram-positive species. Consequently, the gram-positive colony count was significantly increased in the *L. plantarum* group compared to the control group on these days. The gram-negative count, on the other hand, was significantly reduced in the *L. plantarum* group compared to the control group at all time points. Whether the reported changes were due to the *L. plantarum* or

fructooligsaccharide component is unknown as an appropriate placebo control was not included. Importantly, no serious adverse events were reported in either group throughout the course of the study and no cases of sepsis occurred.

H. Allergenicity

Bovine skim milk protein hydrolysate is a component of the medium used to culture *L. plantarum* L-137 in the production of the HK-LP ingredient. Allergy to cow's milk is recognized as one of the most common food allergies due to the presence of a number of milk proteins, which are known to be allergenic or immunogenic to humans (Bush and Hefle, 1996). The major milk protein allergens are the caseins and β-lactoglobulin. HWFC have confirmed that these milk allergens are not present above specified levels in the final HK-LP ingredient. In addition, HWFC contends that no allergenicity issues have arisen as a result of ingestion of the ingredient; however, as bovine milk proteins can be detected at a marginal level (approximately 10 ppm) in the HK-LP ingredient, the statement "contains milk" will appear on the label for the final ingredient. Furthermore, there is no indication from the scientific literature that *L. plantarum* produces allergens.

I. Summary and Basis for GRAS Conclusion

The GRAS determination for the use of the HK-LP ingredient as a food ingredient is based on scientific procedures. The ingredient is composed of 20% HK-LP and 80% dextrin, the latter of which is affirmed as GRAS by the FDA, and therefore, does not pose any safety or regulatory issues. The HK-LP ingredient is intended for use as a food ingredient in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk and milk products, plant protein products, processed fruit and fruit juices, processed vegetables and vegetable juices, soft candy, soups and soup mixes, and sugar substitutes at a maximum level of 150 mg per serving. The maximum use level of 150 mg of the ingredient per serving provides approximately $3x10^{10}$ HK-LP cells per serving. Under the intended conditions of use, the estimated all-user mean and 90th percentile intakes of the HK-LP ingredient by the total population are 0.62 g and 1.14 g/day, respectively (10.6 and 21.6 mg/kg body weight/day, respectively). The corresponding all-user mean and 90th percentile intakes of HK-LP are 0.124 g and 0.228 g/day, respectively (2.12 and 4.32 mg/kg body weight/day, respectively). Additionally, the estimated mean and 90th percentile all-user intakes of HK-LP are approximately equivalent to an intake of $2.9x10^{10}$ and $5.2x10^{10}$ HK-LP cells/person/day, respectively.

The HK-LP ingredient is produced in accordance with cGMP and meets appropriate food-grade specifications. The ingredient is produced *via* culture of *L. plantarum* L-137, following which the bacteria are killed by heat-treatment. Dextrin is added to the HK-LP for formulation purposes at a dextrin to bacteria weight ratio of 4:1. Numerous purification steps, including UHT

sterilization, filtration, dilution/concentration, and magnetic decontamination are applied throughout the manufacturing process to remove all culture media components, processing aids, and impurities. HWFC has established chemical and microbiological specifications consistent with other food-grade materials. Lot samples are routinely evaluated to verify compliance with the specifications. As bovine milk proteins used in the production of the HK-LP ingredient can be detected at a marginal level (approximately 10 ppm) in the final ingredient, the statement "contains milk" will appear on the label for the final ingredient. Although, HWFC contends that no allergenicity issues have arisen as a result of ingestion of the HK-LP ingredient in Japan.

The safety of the HK-LP ingredient under the intended conditions of use is supported by the taxonomy and history of safe consumption of *L. plantarum* and by the available toxicological and human studies. Species belonging to the *Lactobacillus* genus are gram-positive, non-pathogenic, non-spore-forming, and non-motile rods that occur ubiquitously. The species *L. plantarum* is a non-toxicogenic and non-pathogenic bacteria species that is present in a number of commonly consumed foods (*e.g.*, fermented vegetable dishes, pickles, sauerkraut, and in various types of cheeses and fermented sausages). *L. plantarum* L-137 itself was originally isolated and identified from a fermented rice and fish dish that is native to the Philippines, and therefore, has been historically consumed. *L. plantarum* strain 299v also has been consumed in more recent history as a constituent of a fermented oatmeal, fruit, or yogurt drink in parts of Europe. The history of use of *L. plantarum* in foods is widely accepted as safe as reviewed by a number of scientific groups and qualified experts. In addition, *L. plantarum* has been granted QPS status by EFSA in the European Union.

Special safety considerations relevant to the intended use of microorganisms in food include risk of infection, deleterious metabolic activities of the organism, immunological effects, potential for antibiotic-resistance gene transfer, and production of bacteriocins. Based on the general recognition of safety of lactobacilli, the results of human studies on HK-LP and *L. plantarum* addressing these endpoints, and analytical data confirming the absence of toxin production (*i.e.,* the presence of histamine and tyramine in the final product and the production of bacteriocins), the HK-LP ingredient is expected to be of no safety concern with respect to these considerations. In addition, following consumption of the ingredient, HK-LP is expected to share a similar gastrointestinal fate as live preparations of food-grade bacteria based on its intact nature. In other words, HK-LP is not expected to be absorbed, but to transit through the gastrointestinal and excreted in the feces.

The results of animal toxicity studies conducted on HK-LP-based ingredients as well as various live preparations of *L. plantarum* indicated that *L. plantarum* is of low oral toxicity and are supportive of the safety of the HK-LP ingredient for human consumption at the intended levels of use. An acute oral toxicity study conducted with an HK-LP ingredient (consisting of 50% HK-LP and 50% dextrin) demonstrated no acute toxicity or death in mice administered a single

oral dose of 2,000 mg/kg body weight. Short-term oral studies conducted with live preparations of *L. plantarum* further demonstrated that *L. plantarum* at doses of up to 525 mg/kg body weight/day (providing up to 10^{10} CFU/day) did not produce adverse effects on standard parameters of toxicological testing in healthy mice or in mice with colitis. The results of these studies further indicated that *L. plantarum* does not harbor potential for bacterial translocation, including in cases of increased intestinal permeability. Other studies aimed at elucidating the effect of *L. plantarum* consumption in improving the condition of experimental animals in diseased or compromised states demonstrated that repeated oral administration of *L. plantarum* did not negatively affect the frequency of bacterial translocation (by potentially pathogenic bacteria or lactobacillus species) in experimental mice and rats and did not have any adverse impact on the condition in question. In addition, the results of a postnatal developmental study demonstrated that oral administration of *L plantarum* 299v at a dose of $3.0x10^9$ CFU/kg body weight/day in immature rats did not result in overt toxicity and did not adversely impact intestinal barrier function in either neonatal or postnatal rats. Furthermore, the HK-LP ingredient was shown to be non-mutagenic and non-genotoxic in both *in vitro* and *in vivo* validated systems.

The safety information provided in the animal studies is corroborated by information from human studies conducted with the HK-LP ingredient or live preparations of *L. plantarum*. In a double-blind, randomized, placebo-controlled study, no compound-related adverse effects were reported by subjects consuming 50 mg of the ingredient (*i.e.*, 10 mg of HK-LP or $1.15x10^{10}$ HK-LP cells per day) for a period of 12 weeks. Moreover, the HK-LP ingredient has been consumed in Japan as a food ingredient and encapsulated food product since June 2008 and April 2005, respectively, with no reported adverse effects at doses similar to that used in the human study. In addition to this study, other human studies have been conducted in healthy humans as well as in patient population groups using commercially-available preparations containing live *L. plantarum* strain 299v at doses relevant to the intended use levels of HK-LP in food. In these studies, subjects consumed *L. plantarum* at daily doses within the range of $5x10^7$ to $2x10^{11}$ CFU per person for periods of 1 to 12 weeks. The *L. plantarum* preparations, containing heat-killed or live bacteria, were generally well tolerated by all population groups and no adverse events or side effects attributable to the bacterial preparation occurred. As expected, consumption of live *L. plantarum* 299v by healthy subjects resulted in increased fecal counts for the ingested strain and for the general group of lactic acid bacteria (lactobacilli and bifidobacteria). However, fecal counts for all other microorganisms, including those for potentially pathogenic microorganisms, remained unchanged. Importantly, intake of live preparations of *L. plantarum* 299v did not exacerbate underlying conditions or illnesses, such as IBS, and did not result in increased intestinal permeability or incidences of bacterial translocation and septic-complications. Furthermore, given that the intestinal barrier becomes substantially less permeable with increasing postnatal age and maturity and that lactobacilli are not pathogenic and exist as part of the normal intestinal microflora of healthy individuals, including infants and children, the consumption of HK-LP by post-weaning infants and children is not expected to result in adverse effects. Indeed, in one study, feeding of healthy newborn

infants daily with an *L. plantarum* preparation containing 10^9 cells over 7 days did not induce any incidence of sepsis or any serious adverse event. The issue of potential bacterial translocation in those with developing and immature gastrointestinal systems is further not a concern with the HK-LP ingredient as the bacteria are heat-killed.

Together, the above data provided support the conclusion that the consumption of the HK-LP ingredient under the intended conditions of use would not be expected to produce adverse effect in consumers.

Finally, the Expert Panel convened on behalf of HWFC, independently and collectively, critically evaluated the data and information summarized above, and concluded that the intended uses of the HK-LP ingredient, trade named LP20, produced consistently with cGMP and meeting appropriate food grade specifications described herein, are safe and suitable. Furthermore, the Expert Panel unanimously concluded that the intended uses of the HK-LP ingredient, meeting appropriate food-grade specifications and produced consistent with cGMP, are GRAS based on scientific procedures. It is also HWFC's opinion that other qualified scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, HWFC has concluded that the HK-LP ingredient is GRAS under the intended conditions of use on the basis of scientific procedures. The HK-LP ingredient is GRAS based on scientific procedures for its intended uses in food; therefore, it is excluded from the definition of a food additive and thus may be marketed and sold for the uses designated above in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

V. REFERENCES

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
131—Milk and cream	131.200	Yogurt
	131.203	Lowfat yogurt
	131.206	Nonfat yogurt
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
177—Indirect food additives: Polymers	177.1655	Polysulfone resins
	177.2260	Filters, resin-bonded
184—Direct food substances affirmed as generally recognized as safe	184.1277	Dextrin
	184.1763	Sodium hydroxide

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Appendix A

APPENDIX A

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS A HEAT-KILLED *LACTOBACILLUS PLANTARUM* INGREDIENT IN FOOD

EXPERT PANEL CONSENSUS STATEMENT REGARDING THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF A HEAT-KILLED *LACTOBACILLUS PLANTARUM* INGREDIENT FOR USE IN FOODS

February 19, 2009

INTRODUCTION

At the request of House Wellness Foods Corporation (HWFC), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether, under the conditions of intended use as a food ingredient, a heat-killed *Lactobacillus plantarum* (HK-LP) ingredient (trade named LP20), would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of the below-signed qualified scientific experts: Prof. Michael W. Pariza, Ph.D. (University of Wisconsin), Prof. Stephen L. Taylor, Ph.D. (University of Nebraska), and Prof. Gary M. Williams, M.D. (New York Medical College). *Curricula vitae* evidencing the Panel members' qualifications for evaluating the safety of food ingredients are provided in Attachment 1.

The Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other published sources through January of 2009. In addition, the Panel evaluated other information deemed appropriate or necessary, including data and information provided by HWFC. The information evaluated by the Panel included details pertaining to the method of manufacture and product specifications, supporting analytical data, intended use-levels in specified food products, consumption estimates for all intended uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of LP20 and heat-killed or live *L. plantarum*.

Following independent, critical evaluation of such data and information, the Panel convened on 19 February 2009 and unanimously concluded that the intended uses in traditional foods described herein for LP20, meeting appropriate food-grade specifications as described in the supporting dossier [Documentation Supporting the Generally Recognized As Safe (GRAS) Status of a Heat-Killed *Lactobacillus plantarum* Ingredient For Use In Traditional Food Products] and manufactured consistent with current Good Manufacturing Practices (cGMP), are GRAS based on scientific procedures. A summary of the basis for the Panel's conclusion is provided below.

SUMMARY AND BASIS FOR THE GRAS STATUS OF LP20

LP20, HWFC's heat-killed *L. plantarum* ingredient, is composed of 20% heat-killed *L. plantarum* strain L-137 (HK-LP) and 80% dextrin. As dextrin is affirmed as GRAS by the FDA as a direct food substance for use in food for human consumption with no limitation other than cGMP (21 CFR §184.1277) (U.S. FDA, 2008a), the dextrin component of the LP20 ingredient does not pose any safety or regulatory issues. Hence, the safe and suitable use in foods and GRAS status of LP20 relies heavily on the HK-LP component of the ingredient. The taxonomic classification of *L. plantarum* is as follows: Cellular organisms; Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus. *L. plantarum* L-137 is a non-pathogenic, gram-positive, rod-shaped, non-motile, lactic acid-producing bacterium originally isolated from a fermented fish and rice dish that is commonly consumed in the Philippines (Olympia *et al.*, 1992, 1995). Various other *L. plantarum* strains also have been safely consumed on a world-wide basis as a component of many types of fermented foods, including, but not limited to, fermented vegetable dishes, pickles, sauerkraut, and in various types of cheeses and fermented sausages (Orillo and Pederson, 1968; Orillo *et al.*, 1969; Olympia *et al.*, 1992; Rebecchi *et al.*, 1998; Tanasupawat *et al.*, 1998; Baruzzi *et al.*, 2000; Tamminen *et al.*, 2004; Rantsiou *et al.*, 2006, 2008; García Fontán *et al.*, 2007; Plengvidhya *et al.*, 2007). *L. plantarum* strain 299v also has been consumed in more recent history as a constituent of a fermented oatmeal, fruit or yogurt drink in parts of Europe (Molin, 2001). *L. plantarum*, along with other lactobacilli, is a commensal bacterium present as part of the normal intestinal and oral microflora of healthy humans (Molin *et al.*, 1993; Ahrné *et al.*, 1998; Song *et al.*, 2000). Specific exposure to dead lactobacilli occurs through consumption of yogurts that have been heat-treated following fermentation, a practice that is permitted by the FDA to destroy viable microorganisms (21 CFR §131.200, 21 CFR §131.203, and 21 CFR §131.206) (U.S. FDA, 2009).

LP20 is intended for use in foods and beverages, including beverages and beverage bases, breakfast cereals, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk and milk products, plant protein products, processed fruit and fruit juices, processed vegetables and vegetable juices, soft candy, soups and soup mixes, and sugar substitutes. LP20 is intended for use in selected ready-to-eat foods and beverages at use-levels of 0.03 to 0.50%, depending on the food or beverage. In products requiring further preparation prior to consumption (*i.e.*, beverage powders, coffee whiteners, dry soup mixes, and table-top sugar substitutes), use-levels of 0.50 to 7.50% are proposed for LP20.

The consumption of LP20 from all intended food uses was estimated using the National Center for Health Statistics' 2003-2004 National Health and Nutrition Examination Surveys (CDC, 2006; USDA, 2008), which provide the most appropriate data for evaluating food-use and food-consumption patterns in the United States. Under the conditions of intended use of LP20 in food, total U.S. population all-user mean and 90th percentile daily intakes from all proposed food

uses were estimated to be 0.62 g/person/day (10.6 mg/kg body weight/day) and 1.14 g/person/day (21.6 mg/kg body weight/day), respectively.

LP20 is produced in accordance with cGMP and meets appropriate food-grade specifications. Briefly, *L. plantarum* L-137 is cultured, killed by heat treatment, and concentrated, following which dextrin is added to produce the final powdered product. Lot samples are routinely assayed to verify compliance with specifications. Carry-over of raw materials into the final product, particularly of those utilized as components of the culture medium, is prevented at the filtration stage. In addition, downstream steps in the manufacturing process include a final filtration step to ensure removal of aggregated HK-LP, undissolved dextrin, and foreign substance contamination as well as ultra-high temperature sterilization. The final product specifications ensure that HK-LP and dextrin comprise 20% and 80% of the overall composition of LP20, respectively, and analysis of sample lots demonstrate a consistent product. The average protein content in LP20 was determined to be 3.8%, which corresponds to an HK-LP protein content of approximately 20%, and the average nucleic acid content in LP20 was determined to be 2.4%, corresponding to an HK-LP nucleic acid content of 12%. In addition, analysis of the LP20 ingredient for chemical impurities, heavy metals, and microbes indicate conformance to the limits set by the product specifications. The analytical data further demonstrate that residual levels of the raw materials used as components of the culture medium, particularly those for bovine milk proteins, are well below the specification parameters, demonstrating successful removal of these materials during the filtration process. Additionally, the biogenic amines histamine and tyramine were undetectable in the LP20 ingredient upon analysis. Furthermore, all reagents used in the manufacture of LP20 are food-grade materials.

Studies evaluating the acute toxicity and mutagenicity of LP20 or a similar ingredient, as well as tolerance in humans were used to substantiate the safety of LP20 for human consumption. Since LP20 consists of HK-LP, safety data on the oral consumption of live *L. plantarum* retrieved from repeat-dose and developmental studies as well as studies conducted in humans also were reviewed to assess the potential toxicity of HK-LP. The fate of the bacterial species following ingestion also was considered in light of the knowledge that absorption of live bacteria from the gastrointestinal tract, a phenomenon known as bacterial translocation, is an undesirable effect that may lead to bacteremia, sepsis, and multiple organ failure. However, in normal healthy individuals, the intestinal mucosal barrier is impermeable to bacteria. It is in compromised individuals in which bacterial translocation may occur as a result of susceptibility (Ishibashi and Yamazaki, 2001; Lichtman, 2001; Liong, 2008). Furthermore, the potential for HK-LP to translocate from the gastrointestinal tract and cause infection is likely to be non-existent, considering that the bacteria are dead. Instead, HK-LP is expected to transit through the gastrointestinal tract and be excreted in the feces.

The results of animal toxicity studies conducted on HK-LP-based ingredients as well as various live strains of *L. plantarum* are supportive of the safety of LP20 for human consumption at the

intended levels of use. A single oral dose of 2,000 mg/kg body weight of an HK-LP ingredient (consisting of 50% HK-LP and 50% dextrin and named LP20 prototype) did not result in acute toxicity or death in Slc:ICR mice. Thus, an oral LD_{50} of greater than 2,000 mg/kg body weight was established for this HK-LP ingredient in both male and female mice.

Although subchronic toxicity data on LP20 were not available, brief safety assessments were conducted on live *L. plantarum* strains (NCIMB8826 and Lp-115), indicating that short-term repeated oral administration of *L. plantarum* at doses of 10^9 or 10^{10} colony-forming units (CFU)/day was not associated with any compound-related adverse effects in healthy BALB/c mice (Pavan *et al.*, 2003; Daniel *et al.*, 2006a). Specifically, no variations in mouse activity or body weight and no signs of macroscopic or histological inflammation were detected in the ileum or colon of male mice administered *L. plantarum* NCIMB8826 at a dose of 10^9 CFU/day for 4 days (Pavan *et al.*, 2003) or in female mice administered *L. plantarum* Lp-115 at a dose of 10^{10} CFU/day for 5 days (Daniel *et al.*, 2006a) compared to control mice (vehicle only). No neutrophil recruitment in tissues of the colon and small intestine was observed, and mesenteric lymph node, spleen, liver, and kidney cultures were negative for the administered bacteria (Pavan *et al.*, 2003; Daniel *et al.*, 2006a). Even in mice induced with colitis, a mouse model for inflammatory bowel disease, mesenteric lymph node, spleen, liver, and kidney cultures were negative for the administered *L. plantarum* strain, except in one colitic female mouse displaying low levels of *L. plantarum* Lp-115 in the liver and kidneys (Pavan *et al.*, 2003; Daniel *et al.*, 2006a). Additionally, among the healthy groups of mice, both control mice and mice administered *L. plantarum* displayed low numbers of endogenous microflora in these organs. The investigators concluded that feeding live *L. plantarum* did not induce adverse effects or abnormal translocation of the bacteria administered even in mice with increased intestinal permeability.

Similarly no significant differences in food intake, body weight, visceral weight indices for lymph nodes, spleen, and liver, or behavior were observed between male ICR mice orally administered (route not specified) 10^7 CFU/day of *L. plantarum* pH04 for 14 days and control mice not receiving bacteria (Nguyen *et al.*, 2007). Furthermore, the occurrence of bacterial translocation was similar among groups. Other studies reviewed were aimed at elucidating the effect of *L. plantarum* consumption in improving the condition of experimental animals in diseased or compromised states and did not specifically address the safety of oral administration of *L. plantarum* in and of itself. Nonetheless, the results of these studies demonstrated that repeated oral administration of *L. plantarum* did not negatively affect the frequency of bacterial translocation (by potentially pathogenic bacteria or lactobacillus species) in experimental mice and rats and did not have any adverse impact on the condition in question (Mao *et al.*, 1996; Kasravi *et al.*, 1997; Kennedy *et al.*, 2000; Liu *et al.*, 2001; Wang *et al.*, 2001; Schultz *et al.*, 2002; von Bültzingslöwen *et al.*, 2003; Dieleman *et al.*, 2003; Osman *et al.*, 2004, 2007, 2008; White *et al.*, 2006; Håkansson *et al.*, 2006; Han *et al.*, 2006; Mangell *et al.*, 2006; Gross *et al.*, 2008).

No traditional reproductive or developmental toxicity studies were conducted on LP20; however, in a study conducted in neonatal and postnatal Sprague-Dawley rats, administration of *L. plantarum* 299v was shown not to result in any adverse effects (Fåk *et al.*, 2008). Rats were gavaged with 3.0 x 10^6 CFU/g body weight/day of *L. plantarum* 299v for 7 days at ages 3, 7, or 14 days, following which adverse effects of diarrhea, behavioral changes, and body weight gain problems were not observed. Increased macromolecular barrier function of the small intestine was observed in the youngest age group receiving *L. plantarum* 299v, but not in other age groups, compared to control mice given saline. Significant, yet slight, reductions in relative pancreatic and adrenal weight of animals dosed from age 7 to 14 days with *L. plantarum* 299v were not considered adverse, but deemed as reflecting favorable effects in response to the establishment of more efficient intestinal digestive capabilities and a reduction in physiological stress, respectively, as discussed by the authors. A significant, yet slight, reduction in the relative weight of the distal portion of the small intestine was observed in the oldest age group only; however, no other significant changes were observed in the relative organ weights of the proximal small intestine, cecum, liver, stomach, spleen, and thymus, or on the pH of the stomach. The results of this study demonstrate that oral administration of *L. plantarum* 299v in immature rats did not adversely impact intestinal barrier function in either neonatal or postnatal rats.

LP20 did not exhibit any mutagenic potential *in vitro* in standard *Salmonella typhimurium* and *Escherichia coli* bacterial strains at doses of up to 5,000 μg/plate (Hirose *et al.*, 2009). Furthermore, LP20 was shown to be non-clastogenic *in vitro* in a Chinese hamster lung fibroblast cell line at concentrations of up to 5,000 μg/mL, and also did not increase micronucleus frequency in mouse bone marrow cells *in vivo* at doses of up to 2,000 mg/kg body weight (Hirose *et al.*, 2009).

The safety of LP20 was assessed in humans within a double-blind, randomized, placebo-controlled study in which participants ingested one capsule providing either 50 mg of LP20 or dextrin (placebo) daily for a period of 12 weeks (Hirose *et al.*, 2006). No significant changes were detected in anthropometric measurements, clinical chemistry, urinalysis, and hematology results between groups, apart from a significant increase in lymphocyte count in the LP20 group, which remained within the range of reference values. Several adverse events were reported in each group, and none were attributed to the dietary intervention. Additionally, the quality of life score was not adversely affected in the LP20 group compared to baseline and control group scores. In addition to this study, other human studies have been conducted using commercially-available preparations containing live *L. plantarum* strain 299v with the purpose of delineating potential favorable properties of *L. plantarum* consumption in healthy humans as well as in patient population groups. In these studies, subjects consumed *L. plantarum* at daily doses within the range of 5 x 10^7 to 2 x 10^{11} CFU per person for periods of 1 to 12 weeks. The *L. plantarum* preparations, containing heat-killed or live bacteria, were generally well tolerated by all population groups and no adverse events or side effects attributable to the bacterial

preparation occurred. Moreover, no adverse reactions have been reported as a result of supplemental intake of LP20 at a dose of 52 mg in Japan.

In patients diagnosed with irritable bowel syndrome (IBS), a gastrointestinal disorder, ingestion of live *L. plantarum* 299v did not negatively impact symptoms of IBS, such as abdominal pain, irregular bowel movements, and flatulence (Nobaek *et al.*, 2000; Nieldzielin *et al.*, 2001; Sen *et al.*, 2002). Stool frequency and consistency also were unaffected in healthy population groups (Johansson *et al.*, 1998; Goossens *et al.*, 2003, 2005, 2006). As expected, consumption of live *L. plantarum* 299v by healthy subjects resulted in increased fecal counts for the ingested strain and for the general group of lactic acid bacteria (lactobacilli and bifidobacteria). However, fecal counts for all other microorganisms, including those for potentially pathogenic microorganisms, remained unchanged.

In smokers and moderately hypercholesteremic men, parameters related to cardiovascular health, including systolic and diastolic blood pressure, blood lipid levels, and plasma fibrinogen levels, as well as plasma glucose and insulin levels were unchanged or not adversely affected by ingestion of live *L. plantarum* 299v compared to placebo (Bukowska *et al.*, 1998; Naruszewicz *et al.*, 2002).

In other studies conducted in patient population groups (patients with *Clostridium difficile*-associated diarrhea, patients undergoing abdominal surgery, critically-ill hospitalized patients, or hospitalized patients with acute pancreatitis), ingestion of live *L. plantarum* 299v failed to increase intestinal permeability or incidences of bacterial translocation and septic-complications (McNaught *et al.*, 2002, 2005; Wullt *et al.*, 2003, 2007; Klarin *et al.*, 2008; Qin *et al.*, 2008).

Collectively, the available human data on *L. plantarum* indicate that human consumption of the heat-killed or live bacteria at the intended use-levels is not expected to be associated with any adverse effects, especially with regards to translocation of the administered live bacteria, or exacerbate symptoms of underlying illnesses, such as IBS.

LP20 is intended for use in foods that may be consumed by post-weaning infants and children, and therefore, the safety of LP20 in infants and children was specifically addressed as they represent a special population group. Although the human neonatal intestinal barrier is permeable to macromolecules, this is most associated with pre-term infants who are thus susceptible to translocation of pathogenic bacteria and to sepsis as a result of an immature intestinal barrier (Axelsson *et al.*, 1989; Kuitunen *et al.*, 1994; Westerbeek *et al.*, 2006; Anand *et al.*, 2007). With increasing postnatal age and maturity, the intestinal barrier becomes substantially less permeable (Axelsson *et al.*, 1989; Kuitunen *et al.*, 1994). In addition, lactobacilli are not pathogenic and exist as beneficial commensal bacteria present as part of the normal intestinal microflora of healthy humans, including healthy infants and children (Molin *et al.*, 1993; Song *et al.*, 2000; Annuk *et al.*, 2001; Park *et al.*, 2002; Ahrné *et al.*, 1998, 2005; Nguyen *et al.*, 2007). This demonstrates the occurrence of *L. plantarum* as part of the natural

intestinal microflora beginning from as early as the newborn stage. Moreover, a delay in intestinal colonization with beneficial bacteria, such as lactobacilli, may instead increase the susceptibility of pre-term infants to bacterial translocation involving pathogenic bacteria (Van Camp *et al.*, 1994; Westerbeek *et al.*, 2006; Claud and Walker, 2008; Hunter *et al.*, 2008). Indeed, feeding of healthy newborn infants daily with an *L. plantarum* preparation containing 10^9 cells and 150 mg of fructooligsaccharides dissolved in dextrose saline over 7 days did not induce any incidence of sepsis or any serious adverse event (Panigrahi *et al.*, 2008). The issue of potential bacterial translocation in those with developing and immature gastrointestinal systems is further not a concern with LP20 as the bacteria added to the LP20 ingredient are heat-killed.

Considerations specific to the safety of microorganisms proposed for use in food exist and include taxonomy, pathogenicity, potential toxin production, antibiotic-resistance potential, safe history of use in food, reports of adverse events, metabolic considerations, environmental presence, viability, and whether the microorganism is a common resident in the intestinal microflora in conjunction with general aspects of safety (*e.g.*, exposure and method of manufacture) (Mattia and Merker, 2008). These are considered since, theoretically, living microorganisms by nature may produce the following side-effects in susceptible individuals: infection, excessive immune stimulation, deleterious metabolic activities, and gene transfer (Salminen *et al.*, 1998; Marteau and Shanahan, 2003). With regards to these particular safety considerations, several reviews on the safety of *Lactobacilli* have been published presenting evidence in support of the general safety and history of use of microorganisms belonging to this genus (Adams and Marteau, 1995; Saxelin *et al.*, 1996; Salminen *et al.*, 1998, 2002; Adams, 1999; Borriello *et al.*, 2003; Marteau and Shanahan, 2003; Gueimonde *et al.*, 2004; Bernardeau *et al.*, 2006, 2008; Sullivan and Nord, 2006; Snydman, 2008). As such, lactobacilli are generally regarded as safe for use in food and food production within this scientific community. Furthermore, using the Qualified Presumption of Safety (QPS) approach, which places significance on the above considerations, the European Food Safety Authority (EFSA) Scientific Committee concluded that most *Lactobacillus* species can be considered to be non-pathogenic to humans and that there were no specific safety concerns regarding these species (EFSA, 2007). Therefore, the EFSA Scientific Committee granted a number of *Lactobacillus* species, including *L. plantarum*, QPS status based on the long history of safe use of these microorganisms in the food chain. The Panel on Biological Hazards further retained the QPS status of *L. plantarum* in 2008 along with that of many other *Lactobacillus* species (EFSA, 2008). Moreover, *L. plantarum* appears on the International Dairy Federation and the European Food and Feed Cultures Association's inventory of microorganisms with a documented history of use in human food as a result of its long history of use in food without adverse effects (Mogensen *et al.*, 2002). Based on these evaluations and owing to the natural occurrence of *L. plantarum* in commonly consumed foods around the world as described in Section 5.1, it may be concluded that a long history of safe consumption exists for *L. plantarum.*

As reviewed, cases of infection due to lactobacilli, including *L. plantarum*, are rare and have occurred almost exclusively in immunocompromised patients or in patients with severe underlying illnesses, which predisposed them to infection. In most cases, the source of the organism appears to have been the patient's own microflora. The few infections in which probiotic preparations containing lactobacilli have been implicated may have been due to contamination of central venous catheters and intestinal feeding tubes. Moreover, it has been reported that increased consumption of food products containing lactobacilli over the years has not led to increases in infections due to lactobacilli. On a related note, adverse events of an immunological nature as a result of consumption of food products containing lactobacilli have not been reported in humans. Based on the findings of the 12-week human study conducted on LP20 (Hirose *et al.*, 2006), there is no evidence indicating that ingestion of HK-LP would result in infection or adverse immunomodulation in healthy persons. With regards to the intrinsic metabolic activities of lactobacilli that theoretically may have harmful effects, these occur naturally due to the activity of endogenous intestinal microflora, and no clinical diseases due to deleterious metabolic effects of probiotic lactobacilli appear to have been reported. In addition, from the series of studies conducted by Goossens *et al.* (2003, 2005, 2006), consumption of *L. plantarum* strain 299v at a daily dose of 2×10^{11} CFU/person/day did not alter fecal β-glucosidase or β-glucuronidase activity, endotoxin concentration, short-chain fatty acid concentrations, or pH in healthy humans. Furthermore, owing to the fact that HK-LP is non-viable, and therefore, unable to undergo any metabolic functions, the metabolic activities attributed to lactobacilli are likely of no consequence. The latter is supported by the lack of biogenic amine (histamine and tyramine) content in the LP20 ingredient as stated previously. With regards to the transfer of genes that confer antibiotic resistance, certain strains of *L. plantarum* do carry resistance to chloramphenicol, vancomycin, or tetracycline; however, lactobacilli are sensitive to other common antibiotics and, in most instances, the antibiotic resistance gene is intrinsically located within the chromosome of lactobacilli, and therefore, not transferable.

Additional considerations that are relevant to the safety of LP20 use in food include the potential for *L. plantarum* to produce bacteriocins and the potential for incorporation of allergens associated with the ingredient. Bacteriocins are antibacterial substances naturally produced by food-grade lactic acid bacteria, including *L. plantarum*, that inhibit the growth of other bacteria (Eijsink *et al.*, 2002; Cotter *et al.*, 2005; DeVuyst and Leroy, 2007). The main concerns are the potential development of resistance to bacteriocins among target bacterial species and the production of bacteriocins that have similar mechanisms of action to clinically-important antibiotics. Following appropriate testing, however, the HK-LP component of LP20 was shown not to produce antibiotics, and is therefore, not of concern in this respect. Hydrolysates of the milk proteins casein and β-lactoglobulin, which are the major allergens associated with bovine milk allergy, are components of the medium used in the culture of *L. plantarum* L-137. HWFC contends that no allergenicity issues have arisen as a result of LP20 ingestion; however, as bovine milk protein can be detected at a marginal level (approximately 10 ppm) in the LP20

ingredient, the statement "contains milk" appears on the label for LP20 as well as on the label of foods and beverages containing LP20. With regards to allergen production by *L. plantarum*, there is no indication from the literature that *L. plantarum* produces any allergens. Instead *L. plantarum* has been implicated as having immunomodulatory properties with potential for diminishing allergic responses (Murosaki *et al.*, 1998; Repa *et al.*, 2003; Daniel *et al.*, 2006b; Hirose *et al.*, 2006; Hisbergues *et al.*, 2007; Puertollano *et al.*, 2008).

The weight of the scientific evidence presented herein indicates that HWFC's LP20 ingredient is safe for its intended use in food. In summary, LP20 is produced by HWFC in accordance with cGMP and meets appropriate food-grade specifications. LP20 is composed of 20% HK-LP and 80% dextrin, the latter of which is affirmed as GRAS by the FDA as a direct food substance. Following consumption, HK-LP will not translocate given its non-viability. The safety of LP20 is confirmed and supported by a series of animal toxicity and *in vitro* mutagenicity and genotoxicity studies conducted on LP20 or on an ingredient composed of 50% HK-LP and 50% dextrin. In support of the safety of LP20 are animal and human studies assessing the safety and tolerability of live *L. plantarum* preparations at or above dose levels relevant to the conditions of intended use of LP20 in foods. The safety of LP20 is additionally supported by the documented long history of safe consumption of *L. plantarum*. In conclusion, the data and information summarized in this report demonstrate that the intended uses of HWFC's LP20, meeting appropriate food-grade specifications and manufactured in accordance with cGMP, would be GRAS, based on the scientific procedures.

CONCLUSION

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the intended uses of a heat-killed *Lactobacillus plantarum* (HK-LP) ingredient, trade named LP20, meeting appropriate food-grade specifications presented in the supporting dossier [Documentation Supporting the Generally Recognized As Safe (GRAS) Status of a Heat-Killed *Lactobacillus plantarum* Ingredient For Use In Traditional Food Products] and produced consistent with current good manufacturing practices (GMP), are safe and suitable.

We further conclude that the intended uses of the HK-LP ingredient, meeting appropriate food-grade specifications presented in the supporting dossier and produced consistent with current GMP, are GRAS based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

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Appendix B

APPENDIX B

METHODS OF ANALYSES

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Appendix 1

Bacterial content determination

1. Bacterial content is analyzed by using the following the method with N = 2.

(1) Heat a 50 mL polypropylene tube up to constant weight at 105°C, and determine the weight accurately.

(2) Precisely weight 2.0 g of LP20 (sample weight), and put it into the tube from (1).

(3) After adding 20 mL of deionized water and suspending well, centrifuge at 3400 rpm for 15 minutes.

(4) After removing the supernatant, add 20 mL of deionized water, suspend and centrifuge at 3400 rpm for 15 minutes. Repeating this procedure three times.

(5) Remove the supernatant after the final centrifugation, dry the tube at 105°C until it reaches constant weight, and determine the weight accurately (dry substance weight).

(6) Calculated insoluble content from sample weight and dry substance weight corresponds to the bacterial content.

Bacterial content (%) = dry substance weight/sample weight × 100

House Wellness Foods Corporation
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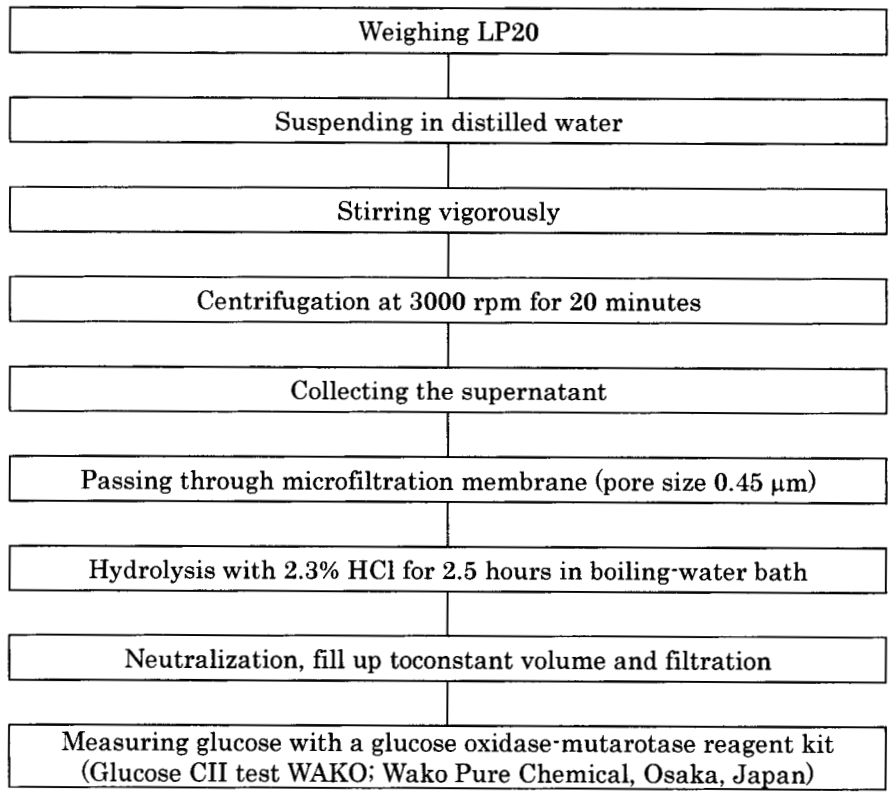
January 9, 2009

Flow chart of method for measuring dextrin content in LP20



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Loss on Drying

The Loss on Drying Test is designed to measure the amount of water and volatile matters in a sample when the sample is dried under specified conditions.

Hereinafter in the Monographs, such a specification as "not more than 0.50 % (105℃, 3 hours)" indicates that when determined by drying 1 to 2 g of the sample, accurately weighed, at 105℃ for 3 hours, the loss in weight is not more than 0.50 % of the sample. Also such a specification as "not more than 0.50 % (0.5 g, not more than 1.3kPa, 24 hours)" indicates that when determined by placing about 0.5 g of the sample, accurately weighed, in a desiccator with silica gel as the desiccant and drying under pressure at 1.3kPa or less for 24 hours, the loss in weight is not more than 0.50 % of the sample.

Procedure Dry a weighing bottle for about 30 minutes under the prescribed conditions, allow to cool it in a desiccator if heated, and weigh it accurately. If the sample is large crystals or lumps, promptly crush it into particles not larger than about 2 mm in diameter and, unless otherwise specified, place 1 to 2 g into the weighing bottle, spread the sample so that the layer is not thicker than 5 mm, and weigh it accurately. Place the bottle in the drying oven, remove the stopper (placing it nearby), dry under the specified conditions, stopper again, take the bottle out of the oven, and weigh it again. If heated, unless otherwise specified, allow to cool it in a desiccator, and weigh it accurately. If the sample melts at a temperature lower than the specified drying temperature, dry it at a temperature 5 — 10℃ lower than the melting temperature for 1 to 2 hours, and dry it under the specified conditions.

Residue on Ignition

The Residue on Ignition Test is designed to measure the weight of the residual substance when sulfuric acid is added to the sample and then ignited.

Hereinafter in the Monographs, such a specification as "not more than 0.10%" indicates that when determined by igniting 1 to 2 g of the sample, accurately weighed, with sulfuric acid at 450−550℃ for 3 hours, the weight of the residue is not more than 0.10% of the sample. Also, such a specification as "not more than 0.02% (5 g, 850℃, 30 minutes)" indicates that when determined by igniting about 5 g of the sample, accurately weighed, with sulfuric acid at 850℃ for 30 minutes, the weight of the residue is not more than 0.02% of the sample. When the stipulation "dried substance" is given in the Monographs, the test is performed, using the sample dried under the conditions specified under Loss on Drying in the individual monograph.

Procedure Ignite a crucible of platinum, quartz, or porcelain under the conditions specified in the individual monograph for about 30 minutes, allow to cool in a desiccator, and weigh it accurately.

If the sample is large crystals or lumps, quickly crush it into particles not larger than about 2 mm in diameter and, unless otherwise specified, place 1 to 2 g of the particles in the crucible described above, and weigh it accurately. Moisten the sample with a small amount of sulfuric acid, then ignite slowly at a temperature as low as practical until the sample is almost incinerated, and allow to cool. Add again 1 ml of sulfuric acid, and heat slowly until white fumes are no longer evolved. Transfer it into an electric furnace, and, unless otherwise specified, ignite it at 450−550℃ for 3 hours. Cool the crucible in a desiccator and weigh it accurately. When the amount of the residue so obtained exceeds the limit specified in the individual monograph, ignite to constant weight.

Inductively Coupled Plasma-Atomic Emission Spectrometry

Inductively coupled plasma-atomic emission spectrometry is designed to determine the amount (concentration) of a test element in a sample, by atomizing and exciting the element by inductively coupled plasma (ICP), and determining the intensity of atomic emission spectral line.

Apparatus Usually, the apparatus consists of an excitation source part, a sample introduction part, a light emission part, a spectroscope part, a photometry part, an indication and recording part. The excitation source part is composed of an electric power source, a control system, and circuit to supply and control the electric energy which excites and emits an element in a sample. This part also includes a gas source and a cooling system. The sample introduction part is composed of a nebulizer and a spray chamber. The light emission part is composed of a torch and an high-frequency induction coil. The spectroscope part is composed of a light-converging system and a spectroscope such as a diffracting grating. The photometry part is composed of a detector and a signal processing system. The indication and recording part is composed of a display and a recording system. The ICP-atomic emission spectrometry includes single-element-sequential-type- and multiple-element-sequential-type-measuring methods using a wavelength scanning spectroscope, and a simultaneously measuring method using a wavelength-fixed-type polychrometer.

Procedure Confirm that all live parts are normal. Switch on the excitation source part and the cooling system. When a vacuum-type spectroscope is used to measure the emission line in vacuum-ultraviolet region, purge sufficiently the light-path between the light emission part and the spectroscope with argon or nitrogen gas. Set the flow rate of argon or nitrogen gas to the specified rate, switch on the high frequency power, and generate the plasma. Correct the wavelength of spectroscope with the emission spectral line of a mercury lamp. Introduce the test solution and the standard solution or control solution prepared as specified in the individual monograph and measure the emission intensity of an appropriate emission line of the object element.

Usually, the determination is done using one of the following methods. In the determination, the interference and background should be corrected.

(1) Calibration Curve Method Prepare standard solutions of three or more different concentrations, measure the emission intensities of these standard solutions, and prepare a calibration curve from the obtained values. Then, measure the emission intensity for the test solution with a concentration adjusted to a measurable range, and determine the amount (concentration) of the object element from the calibration curve.

(2) Standard Addition Method To equal volumes of three or more test solutions, add to each the standard solution so that the stepwise increasing amounts of the object element are contained in the solutions, and add the solvent to make a definite volume. Measure the emission intensity for each solution, and plot the amounts (concentrations) of added standard object element on the abscissa and the emission intensities on the ordinate on the graph paper. Extend the calibration curve obtained by linking the plots, and determine the amount (concentration) of object element from the distance between the origin and the intersecting point of the calibration curve on the abscissa. This method is applicable only when the calibration curve drawn as directed in section (1) above is a straight line passing through the origin.

(3) Internal Standard Method Prepare several solutions containing a constant amount of the specified internal standard element, and known graded amounts of the standard object element. For these solutions, measure the emission intensities of the standard object element and internal standard element at the analytical wavelength of each element under the same measuring conditions, and obtain the ratios of each emission intensity of standard object element to the emission intensity of the internal standard element. Prepare a calibration curve by plotting the amounts (concentrations) of standard object element on the abscissa and the ratios of emission intensity on the ordinate. Then, prepare the test solutions, adding the same amount of internal standard element as in the standard solution. Proceed under the same conditions as for preparing the calibration curve, obtain the ratio of the emission intensity of standard object element to that of internal standard element, and determine the amount (concentration) of the object element from the calibration curve.

Note: For this test, avoid the use of reagents, test solutions, and gases which interfere with the determination.

Atomic Absorption Spectrophotometry

Atomic Absorption Spectrophotometry is designed to determine the amount (concentration) of an object element in a sample, utilizing the phenomenon that the atoms in the ground state absorb the light of characteristic wavelength passing through an atomic vapor layer of the element.

Apparatus Usually the apparatus consists of a light source, a sample-atomizer, a spectroscope, and a photometer, and a recording system. Some are equipped with a background compensation system. For the light source, a hollow cathode lamp and a discharge lamp are mainly used. To the sample-atomizer, the flame type, electrothermal type, and the cold-vapor type are applied.

The cold-vapor flameless type is categorized as the two methods: reduction vaporizing method and heat vaporizing method. The flame type is composed of a burner and a gas-flow regulator, the electrothermal type is composed of an electric furnace and a power source, and the cold-vapor type is composed of a mercury generator by chemical reduction-vaporization and thermal reduction-vaporization and an absorption cell. For the spectroscope, a grating for light diffraction or an interference filter prism is used. The photometer mainly consists of a detector and a signal treatment system. A recording system is composed of a display and a recording device. A background compensation system is employed for the correction of matrix effects on the measuring system. Several principles can be utilized for background compensation, using the continuous spectrum sources, the Zeeman split spectrum, the nonresonance spectrum, or the self-inversion phenomena.

Procedure Unless otherwise specified, proceed by either of the following methods:

(1) Flame Type Fit the specific light source lamp to the lamp housing, and switch on the instrument. Light the source lamp, adjust the wavelength dial of the spectroscope to the wavelength of the analytical line specified, and set at an appropriate current value and slit-width. Using the supporting gas and combustible gas specified, ignite the mixture of these gases, adjust the gas flow rate and pressure, and make the zero adjustment after nebulizing the solvent into the flame. Nebulize the test solution or the standard solution or control solution prepared by the method prescribed elsewhere, and measure the absorbance.

(2) Electrothermal type Fit the specific light source to the lamp housing and switch on the instrument. After lighting the lamp and selecting the analytical wavelength specified in the individual monograph, set an appropriate electric current and slit-width. A suitable amount of sample solution, standard solution, or control solution, prepared as specified in the individual monograph, is injected to the furnace and an appropriate stream of inert gas is made to flow through the furnace. The

specimen is dried and ashed, and the element included is atomized, on heating at appropriate temperature for an appropriate time in appropriate mode. The atomic absorption specified is observed and the intensity of absorption is measured.

(3) Cold-vapor Type Fit the light source lamp specified on the photometer. Light the source lamp, adjust the wavelength dial of the spectroscope to the wavelength of the analytical line specified, and set at an appropriate current value and a slit-width. Then, in the reduction vaporizing method, transfer the test solution or the standard solution or control solution to the closed vessel, reduce to the element by addition of a proper reducing agent, and vaporize. In the heat vaporizing method, vaporize the sample by heating. Measure the absorbance of the atomic vapor generated by these methods.

Usually, the determination can be done by an appropriate one of the methods given below. In the determination, the interference and background should be considered.

(1) Calibration Curve Method Prepare standard solutions of at least three different concentrations, measure the absorbances of these standard solutions, and prepare a calibration curve from the obtained values. Then measure the absorbance for the test solution adjusted in concentration to a measurable range, and determine the amount (concentration) of the object element from the calibration curve.

(2) Standard Addition Method To equal volumes of more than 2 of different test solutions, add the standard solution so that the stepwise increasing amounts of the object element are contained in the solutions, and add the solvent to make a definite volume. Measure the absorbance for each solution, and plot the amounts (concentration) of added standard object element on the abscissa and the absorbances on the ordinate on graph paper. Extend the calibration curve obtained by linking the plots, and determine the amount (concentration) of object element from the distance between the origin and the intersecting point of the calibration curve on the abscissa. This method is applicable only in the case that the calibration curve drawn as directed in (1) above passes through the origin.

(3) Internal Standard Method Prepare several solutions containing a constant amount of the prescribed internal standard element, and known, graded amounts of the standard object element. Using these solutions, measure the absorbances of the standard object element and the absorabance of internal standard element at the analytical wavelength of each element under the same measuring condition, and obtain the ratios of each absorbance of standard object element to the absorbance of the internal standard element. Prepare a calibration curve by plotting the amounts (concentrations) of standard object element on the abscissa and the ratios of absorbance on the ordinate. Then prepare the test solutions, adding the same amount of internal standard element as in the standard solution. Proceed under the same conditions as for preparing the calibration curve, obtain the ratio of the absorbance of

standard object element to that of internal standard element, and determine the amount (concentration) of the object element from the calibration curve.

Note: For this test, avoid the use of reagents and test solutions which can interfere with the determination.

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Methods for detecting number of bacteria, *Salmonella*, yeast and mold, and coliforms

1. Number of bacteria

(1) Apparatus, instruments, media and dilution water

(a) Apparatus and instruments

Incubator: 35 ± 1 °C
Thermostat bath: 45-50 °C
Balance
Blender with cup for emulsions or stomacher with plastic bag for emulsions
Colony counter
Tweezers
Sterilized scissors

(b) Media

Standard agar: (Nissui, Eiken, Kyokuto, Difco, BBL, Merck)

(c) Dilution water

Physiological saline or 0.1 peptone added physiological saline

Peptone	1 g
Sodium chloride	8.5 g
Purified water	1000 ml

pH 7.0 ± 0.1

Phosphate buffer:

after dissolving potassium dihydrogen phosphate (anhydrous) 34 g in purified water 500 ml, add approximately 175 ml of 1N sodium hydroxide solution, and the total volume is adjusted to be 1000 ml with purified water, and the pH is adjusted to be 7.2. This should be the stock solution and keep in a refrigerator. Add 1 ml of this stock solution to 800 ml distilled water for use.

(2) Preparation of sample stock solution and procedures

For a liquid sample, shake the sampling bottle 25 times or more to mix. For solids, powders and highly viscous samples, weigh a certain amount on a balance, add dilution water and mill with a blender or stomacker to make a uniform sample. Because bacteria in food exist in the Druse condition, homogenize it to disperse piece by piece completely. The sample size is generally 10 g or 10 ml, however, it is designated in the law to be 200 g for oysters to be eaten raw, 25 g for frozen food, and 10 g (10 ml) for other samples (Table 1-8). The sample size differs depending on the country and is not unified: FDA manual 25 g, ISO 10 g and AOAC 50 g.

By the law, sterilized physiological saline, or sterilized phosphate buffer is used for dilution water. Dilution water and added amount differ depending on the type of food, and they are shown in Table 1-8. For frozen foods, bacteria are damaged by the freezing process, and since they are easily killed by physiological saline, phosphate buffer, 0.1% peptone added physiological saline or 0.1% peptone water is advisable. When discriminating dilution water depending on food, unified use of either phosphate buffer or 0.1% peptone added physiological saline avoids confusion.

(3) Test methods

(a) Dilution and pouring

Dilute the sample stock solution by diluent up to 10-fold, 100-fold, 1000-fold, 10000-fold, and if necessary, 100000-fold and 1000000-fold. Diluent which can obtain 30-300 colonies per plate should be selected, however bacterial contamination differs with each food, therefore, 10-fold

serial diluents up to 10^6-fold are prepared generally. Dispense 1 ml of each diluent by using 2 plates for each dilution grade. Pour pre-incubated standard agar media 15-20 ml at 45-50 °C to each dish aseptically, and immediately mix gently to blend the sample and media. On this occasion, the used diluent, media, dishes and pipettes should always be aseptic. To confirm the aseptic condition, take 1 ml of used diluent instead of the sample and add to a plate in which agar medium is added and mixed, or agar only. Operation from sampling and dispensing of media should be within 20 minutes, and diluent-inoculated dishes should not be left for a long time.

For aerobic value count (excluding lactic acid bacteria) of lactic acid bacteria-added powder beverages, 1.0 μg/ml penicillin G potassium-added glucose-added agar media (peptone 10 g, yeast extract 3 g, glucose 5 g, sodium chloride 5 g, agar powder 13 g, purified water 1000 ml, penicillin G potassium 1 mg) is used instead of standard agar media.

(b) Incubation

When agar media are completely coagulated, invert the dishes, and incubate in an incubator at 35 ± 1 °C. Generally, incubation time should be 48 ± 3 hours. However, in cases for which incubation temperature and time are legally specified, they should be followed, in principle. In general, 24-hour incubation is not long enough to understand the accurate viable bacteria count thus 48-hour incubation is desirable.

(c) Calculation of colonies

After incubation for a certain time, take out the dishes from the incubator and count the number of colonies by using a colony counter. In cases where colonies cannot be counted immediately, preserve the dishes in a refrigerator at 5 °C, and calculate within 24 hours. If more than 1 day has passed, the number of growing bacteria may change and accurate results cannot be obtained. Colonies should be calculated as follows.

1) When 30-300 colonies are found per plate

Select the plate with 30-300 colonies per plate and count.

2) When over 300 colonies are formed in all plates

According to the dense colony plate counting method, count the plates of highest dilution ratio.

Dense colony plate counting method: Count the colony within the range by using a tally board with a 1 cm^2 compartment. When there are less than 10 colonies per 1 cm^2, 6 sites vertically pass through the center, and 6 sites at a right angle, a total of 12 sites of colonies should be counted. Avoid overlapping the sites already counted. Find the mean number of colonies in 1 cm^2, multiply this by the dish area and calculate the estimated number of colonies per plate. Multiply 65 by the mean number of colonies pr 1 cm^2 obtained, for 9 cm across dishes.

When there are more than 10 colonies per 1 cm^2, in the same manner as the above, count the colonies in 4 compartments, find the mean number of colonies per 1 cm^2, multiply this by dish area to calculate the number of bacteria.

3) When there are less than 30 colonies in all plates

Count the colonies of lowest dilution ratio. The expression of the number of bacteria should not be the actual count, but describe as below 300 for 10-fold dilution, below 3000 for 100-fold dilution for the sample stock solution.

4) When colonies are diffused, count the corresponding portions, limited to the following conditions.

a) Other colonies are also diffused and diffused colonies do not interfere with counting.

b) When diffusion of colonies is less than a half the plate

5) In the following situation, describe as a laboratory accident (LA).

a) When generation of colonies cannot be confirmed. However, sterilized products or corresponding processed or heated foods shall not be applied.

b) When diffusion of colonies exceeds half the plate.

c) When colonies are found in a control plate (e.g., diluent added plate) which is obviously contaminated.

000089

d) Others considered inappropriate.

(d) Description of bacterial count

For description of bacterial count, arithmetic mean should be determined for the number of colonies in 2 plates of the same dilution ratio, and multiply this by the dilution ratio. Moreover, round off the to two decimal places the obtained figure, add a zero to the one decimal place, and find the bacterial count per 1 g (1 ml) of food. The results found in accordance with the standard plate viable bacterial count should be described as 16000/g (ml) or 1.6×10^4/g (ml).

2. *Salmonella* species

(1)Media, Instruments, Serum

1) Media

Pre-enrichment media: EEM broth (Nissui, Eiken), Lactose broth (BBL, Oxoid), Buffered peptone water (BBL, Oxoid)

Enrichment media: Selenite cystine (SC) broth (BBL, Oxoid), Selenite brilliant green (SBG) broth (Nissui), Rappaport broth (Oxoid, Merck), Hajna tetrathionate medium (Nissui, Eiken, BBL, Oxoid, Merck), SBG sulphur medium (Eiken)

Isolation media: MLCB agar medium (Nissui, Oxoid), Brilliant green agar medium (Nissui, Eiken, BBL, Oxoid)

Biochemical test media: TSI agar medium, LIM medium, SIM medium, Lysine carbohydrate medium, Phenylalanine malonate medium, VP semi-solid agar medium, Simmons citrate medium, etc.

2) Instruments

Kits

Biochemical test kits: (ONPG, oxidase test, etc.)

Rapid test kits: Salmonella 1-2 test (BioControl): ELISA method (Organon Teknika, Dynatek, Nihon Transia); Serobact Salmonella (Disposable Product), Colour Salmonella Test (Wellcome),: DNA probe method (Gene Trak System); Fluorescent antibody method (Clinical Sciences Inc.), etc.

Rapid test kits are not essential, but are shown for reference. For more information, refer to section 8) Rapid tests for Salmonella.

3) Serum

O antigen testing serum (Denka Seiken) and H antigen testing and co-induction serum (Denka Seiken) are shown in Tables 1-14 and 1-15.

(2)Sampling and sample preparation

Meat and meat-processed foods, eggs, milk and dairy products, cereals, coconuts, chocolate, other animal organ-derived agents, and feed are examples of food that are the target of Salmonella testing. In Japan, we have customarily taken one sample from one lot to test, but the ICMSF (International Commission on Microbiological Specifications on Foods) recommends a sampling method involving the idea of lots based on probability. The sampling amount for one sample is at least 50 g or 50 ml. If the sample is solid, sampling should be done aseptically and blended with a stomacher after adding 10 times its volume of liquid medium.

Table 1-14 O Group Testing Serum

O multivalent	O1 multivalent
O2 (A) group	O11 (F) group
O4 (B) group	O13 (G1-2) group
O7 (C1) group	O6, 14 (H) group
O8 (C2) group	O16 (I) group
O9 (D1) group	O18 (K) group
O9, 46 (D2) group	O21 (L) group

O3, 10 (E1-3) group O1, 3, 19 (E4) group	O35 (O) group

Table 1-15 H Antigen Testing Serum and Co-induction Serum

H serum (for testing)	a, b, c, d, e.h, G, i, k, L, r, y, e.n, e, v, w, z_{13}, z_{18}, z, 5, 6, 7, z_6, f, m, p, q, s, t, u, z_{23}, z_{24}, z_{32}, x_3, z_{15}, 2, z_4, z_{10}, z_{29}
H serum (for co-induction)	a, b, c, d, e.h, g, i, k, L, r, y, e.n, I, z, z_4, z_{10}, z_{29}

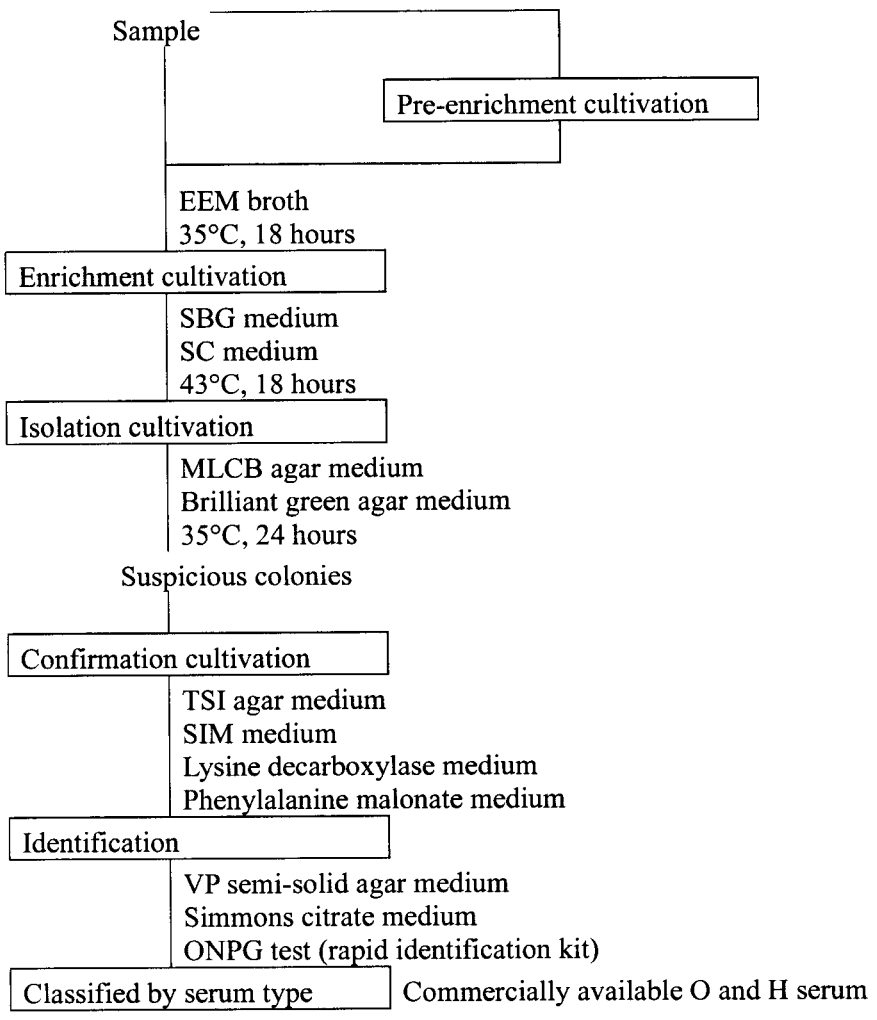

Figure 1-15 Test method of Salmonella

(3) Test method

The key point of the test is that pre-cultivation is necessary before enrichment cultivation since Salmonella in food may be damaged or in hibernation after heating, drying, irradiation and/or freezing. A general outline of the test method is shown in Figure 1-15.

1) Pre-enrichment cultivation

EEM broth, lactose broth and buffered peptone water are used as pre-enrichment media, but all have no selectivity for Salmonella. Add 25 g of prepared sample to 225 ml of pre-enrichment medium and incubate at 35°C for 18 hours. It is convenient to do this step in a stomacher bag. Occasionally mix if possible. Naturally, if the sampling amount increases, the detection rate will also rise.

2) Enrichment cultivation

For samples that do not need to consider damaged bacteria, the number of days required to detect bacteria can be shortened by at least one day because testing can start with enrichment cultivation.

Media such as Selenite cystine medium, Selenite brilliant green medium, Hajna tetrathione medium, and Rappaport medium may be used as enrichment media, but there is no significant difference in accuracy. Add 0.4% Tergitol-7 for samples high in fat such as meat. Incubate for 18 hours after adding 25 g of sample to 225 ml of growth media, and the temperature of the water bath is 37°C for Ravvaport media but 43°C for all other media. Use SBG sulphur medium for eggs and egg products. If pre-enrichment cultivation is done, inoculate 1.5 ml of cultured medium to 15 ml of enrichment medium in a test tube. In particular, the amount of media and temperature must be considered. Specifically, when media is in the container, the depth should be about 7 cm or more. For example, 15 ml is necessary for a test tube (18 mm × 180 mm) and 150 ml is required for a Meyer flask (200 ml). In addition, for incubation at 43°C, a water bath rather than an incubator is recommended. The reason for this is to increase selectivity by decreasing the time required to reach 43°C. After incubation, mix well before proceeding to the isolation cultivation. Cultivation in enrichment media for more than 24 hours may unintentionally result in rapid growth of microbes other than Salmonella, leading to complexities in isolation cultivation.

3) Isolation cultivation

MLCB agar medium, brilliant green agar (BGA) medium, XLD medium, DHL medium, Hektoen medium are customarily used as isolation media, but every technician has a favourite. From our experience, we recommend the simultaneous use of MLCB agar medium, which has high selectivity, and brilliant green agar media, which has low affinity.

Apply one platinum loop of enrichment medium on the agar plate surface and incubate at 35°C for 24 hours. Among isolation media, Salmonella cultivation in MLCB medium leads to generation of hydrogen sulphide and black colonies. However, it is not possible to distinguish between Citrobacter and Proteus, and thus it is necessary to inoculate the initial confirmation media (TSI agar medium, LIM medium, Simmons citrate buffer medium) with as many colonies as possible. Please note that MLCB agar medium, which uses hydrogen sulphide as a marker, is not appropriate for Salmonella that are the causative bacteria for human typhoid, such as *Salmonella* serotype Typhi, *Salmonella* serotype Paratyphi A and *Salmonella* serotype Cholerae-suis. Incubate at 35°C for 18 to 20 hours in confirmation media.

4) Identification of Salmonella

Biochemical characteristics should take precedence in the identification of Salmonella since relying on serum reactions (test aggregation reactions) may result in mistakes. The results for the initial confirmation test are shown in Table 1-16. The most important point is the bright red color on the slant of TSI agar medium. As long as this is remembered, even if Simmons citrate buffer medium is omitted, it is possible to unmistakeably identify common Salmonella. Those that clearly appear purple in the top layers of LIM media are identified as positive, whereas those that are purple in the upper layers are considered negative. The results for this test are also important. Non-hydrogen sulphide-generating and lysine-negative Salmonella strains are excluded from this test, but these will also show the characteristic TSI agar medium slant and even if these are missed due to inexperience, it is very rare for such atypical salmonella to be found in food. Tests required for the secondary confirmation tests and their characteristics are shown in Table 1-17.

Salmonella can be classified into 6 subspecies by its biochemical properties. Subspecies III is ONPG-positive and considered separately from common Salmonella. Most Salmonella derived from humans are subspecies I. In other words, there is a close relationship between subspecies and carcinogenicity. However, *Salmonella* serotype Sofia, subspecies II, is isolated not only in chickens but also in humans. The identification of subspecies is shown in Table 1-18.

Table 1-16 Initial confirmation test for Salmonella

Media		Reaction
TSI	slant	red
	top layer	black
	(hydrogen sulphide)	+
	gas	+
LIM	lysine	+
	indole	-
	motility	+
	IPA	-
Simmons citrate medium		+

Table 1-17 Characteristics seen in the secondary identification stage

Test	Result (%+)
ONPG	- (3)
Lysine	+ (99)
Ornithine	+ (96)
Mannitol	+ (99)
VP	- (0)

Table 1-18 Subspecies of Salmonella

Test	Subspecies			
	I	II	III	IV
Dulcitol	+	+	-	-
Lactose	-	-	+/*	-
ONPG	-	-/*	+	-
Salicin	-	-	-	+
d-Tartrate	+	-/+	-/*	-/*
Mucic acid	+	+	d	-
Malonic acid	-	+	+	-
Gelatin	-	+	+	+
KCN	-	-	-	+

(F. Kauffmann, 1966)

*: By chance or inconsistently positive, d: inconsistent

3. Yeast and mold

(1) Instruments, materials media and reagents

Instruments for sample preparations are common with those for bacteria, therefore they are omitted.

Dish [glass (90 mm × 20 mm) or sterilized plastic (90 mm × 20 mm) are used]

Cotton plug or paper plug, silicone plug for microorganisms (if stainless steel or aluminium cap, should not be loose)

Incubator (usable at low temperature, and the temperature is adjusted to 23-25 °C)

Solid sample grinder or coffee mill

Diluent: 0.1% peptone water, physiological saline (salt 0.85%), 1/4 concentrated Ringer solution, phosphate buffer or distilled water (if necessary, add Tween80 (0.05%). For xerophilic bacteria, 20% saccharose or glucose may be added in order to avoid damage due to osmotic pressure. To aid dispersion of the sample, 0.05% agar may be added)

Media, reagents: For the media for fungus tests, generally, antibiotics are added to suppress the growth of bacteria which can interfere with determination. In general, chloramphenicol (50-100 μg/ml) and gentamicin (50-60 μg/ml) are used due to their high thermostability thus they can be added prior to autoclave sterilization. For other antibiotics with low thermostability, an ezymotized solution purified through a membrane filter is added to the sterilized media.

The next consideration is the fast growing molds, such as zygomycetes (e.g., *Mucor*, *Rhizopus*), which cover the colony of slow growing fungi and interfere with growth. To suppress the growth rate, from the aspect of aerobic viable count, do not hamper its growth completely but it is necessary to add drugs to make it easier to determine as uniform small-sized colonies. For this purpose, rose Bengal (20-150 μg/ml) or dicloran (2,6-dichloro-4-nitroaniline) (2 μg/ml) are added.

The following media are used regularly.

Potato dextrose agar (PDA)

Commercial dry media are available. Dicloran (5 μg/ml) is added to the commercial PDA for dicloran-added (PDA-D5).

Malt extract agar (MEA)

Commercial dry media are available.

Dicloran-rose bengal-chloramphenicol agar (DRBC)

Commercial dry media are available.

Glucose-yeast extract agar (GYA)

Yeast extract	5 g
Glucose	20 g
Chloramphenicol	100 mg
Agar	15 g
Distilled water	1000 ml

pH 6.6 ± 0.1

YM agar (YMA)

Commercial dry media are available.

The following media are used regularly for counting and isolation of xerophilic bacteria.

MY20 agar (MY20)

Peptone	5 g
Yeast extract	3 g
Malt extract	3 g
Glucose	200 g
Agar	20 g
Water	1000 ml

pH 6.0-6.4

Dicloran-18% glycerin agar (DG18)

Peptone	5 g
Glucose	10 g

000096

Potassium dihydrogenphosphate (KH_2PO_4)	1 g	
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5 g	
Dicloran	2 mg	(dissolved in ethanol and added)
Glycerin	220 g	
Chloramphenicol	100 mg	
Agar	15 g	
Water	1000 ml	

pH 5.6 ± 0.2
Aw: 0.955

Commercial dry media (Oxoid) are available.

M40Y agar (M40Y)

Malt extract	20 g
Yeast extract	5 g
Saccharose	400 g
Agar	15 g
Water	1000 ml

pH 5.5-5.9

Malt extract-yeast extract-50% glucose agar (MY50G)

Malt extract	10 g
Yeast extract	2.5 g
Agar	10 g
Add water	500 g
Glucose	500 g

Add malt extract, yeast extract and agar to water 450 ml and heat, and after dissolution, add water to adjust it to be 500 g, and add glucose while it is still hot. Steam sterilize without adding pressure for 30 minutes. Be careful to avoid overheating. Aw: 0.89

Malt extract-yeast extract-5% (or 10%) salt-12% glucose agar (MY5-12 or MY10-12)

Malt extract	20 g	
Yeast extract	5g	
Salt	50 g	(or 100 g)
Glucose	120 g	
Agar	20 g	
Add water	1000 ml	

For sterilization of MY5-12, use an autoclave for 10 minutes at 121 °C. For MY10-12, steam sterilization without adding pressure for 30 minutes. Be careful to avoid overheating. Aw: 0.93 (MY5-12), 0.88 (MY10-12)

Drugs for surface sterilization

75% ethanol

0.1% sodium hypochlorite solution (available chlorine concentration 1000 ppm)

(2) Sampling and sample preparation

This method is applied to various foods and the sampling method is completely the same as that of bacteria. Depending on the food, containers in which the samples are kept should noted, and paper bags are often required to be used for crops, including cereals, vegetables and fruits to avoid becoming musty. Although the growth of fungi takes days, it is desirable to keep the samples in a refrigerator in cases where the test is planned for a future time. However, samples other than frozen food should not be frozen even though they are refrigerated. This avoids the effects of extinction and damage due to freezing.

Although it depends on the type of food, the sample size is generally 50-250 g.

For sample preparation, dry sold foods such as cereals, nuts and oilseeds should be grinded by a sample grinder or small coffee mill to a fine powder in advance. The sample case of the grinder should be sterilized by ethanol immediately before use. Samples which can be readily grinded by a blender do not need to be crushed. Separation of fungi that has invaded into the inside cereals and nuts requires short-time immersion of the sample in ethanol before crushing, and then immersion in sodium hypochlorite for 2 minutes for surface sterilization. After treatment, samples are rinsed with sterile water and placed on the sterilized filter paper to dry.

Although the preparation method of the sample solution is performed similar to bacteria,

excessive blending should be avoided and stopped within 2 minutes due to the reasons such as the hyphae of the fungi can be damaged. Blending by stomacher requires 2 minutes for powdered samples. Some foods have prominent fizziness which requires the use of flasks with glass beads for mixing. A 200 ml Erlenmeyer flask requires 50-100 glass beads (approximately 6 mm across).

(3) Test methods

1) Weigh 10 g of the sample, add 90 ml of diluent and prepare uniform sample stock solution (first suspension) by a homogenizer. If the sample is liquid, mix well, measure 10 ml with a sterilized pipette, and prepare the sample stock solution in the same manner. Prepare 10-fold serially diluted samples from this sample stock solution. The dilution ratio should be prepared so as to be able to obtain 10-100 colonies per plate. In general, dilution requires up to 10^{-4}.
2) Shake each diluent, and apply 1 ml of each diluent to 3 dishes by a pipette.
3) Pour approximately 15 ml of PDA (after melting, heat up to 45 °C) to the above dishes. Immediately, rotate the dishes, and mix the diluent of the sample and media well.
4) For dry foods, use media for xerophilic bacteria counting in combination.
5) After agars are set, turn the dishes upside down, and incubate for 5-7 days, or longer on occasion, at 23-25 °C.
6) After incubation, count all colonies on the plates for which 10-100 colonies are obtained. Extremely small colonies or suspicious colonies should be confirmed microscopically.
7) The above 2) to 6) can be altered to as follows. Pour media to the dishes first, let them set to the plates, apply each 0.5 ml of diluent for 3 agar media, respectively. Apply to the plate surface evenly with a glass spreader. The plates in which diluent of the sample is applied is left on a clean bench for drying of the surface, and then transferred to incubation. Drying of the surface of the medium plates may also be performed by leaving face upward in an incubator at 37 °C overnight with a cover, and after drying, turn over the dishes again and then transfer to 23-25 °C.
8) Find the mean value of the obtained number of the colonies, and calculate the number of fungi in 1 g (1 ml for liquid) by converting from the dilution ratio.

4. Coliforms

(1) Instruments, dilution water, media and reagents

(a) Instruments used

Dry heat sterilizer: 180 °C
Autoclave: 121 ± 1 °C
Incubator: 35 ± 1 °C
Constant temperature water bath: 44.5 ± 0.2 °C
Balance: capable of weighing 200 g at the maximum within the error range of ± 0.1 g.
Blender: capable of blending 100-300 ml, replaceable with Stomacher® 400 Circulator.
Test tubes: medium-size test tube 18 mm × 170 mm
Small test tube 13 mm × 100 mm
Durham tube: applicable to each of the above test tubes.
Measuring pipette: authorized with a nozzle of 1.0 and 10.0 ml, and replaceable with milk pipette (2.2 ml).
Dish: deep and shallow, replaceable with commercial sterilized plastic dishes.
Sample preparation bottle: wide-mouth with ground stopper and gradation marks of 50 and 100 ml.
Culture bottles: 300 ml, 500 ml and 1000 ml of hard glass.
Dilution bottle: for 10-fold dilution; replaceable with medium-size test tube.

(b) Dilution water

Use any of the following.
Peptone / physiological saline

Peptone	1 g
Sodium chloride	8.5 g
Purified water	1000 ml

pH 7.0 ± 0.1
Use after sterilization for 15 minutes at 121 °C.
Phosphate buffered saline
Composition and preparation method: after dissolving potassium dihydrogen phosphate (anhydrous) 34 g in purified water 500 ml, add 1N sodium hydroxide solution approximately 175 ml, adjust the pH to be 7.2 and the total amount is made up to 1000 ml with purified water. This should be the stock solution. Add this stock solution 1.25 ml to physiological saline (sodium chloride 8.5 g dissolved in purified water 1000 ml) 1000 ml, and use it after sterilization for 15 minutes at 121 °C.

(c) Media

Prepare commercial powder media by following the prescriptions.
BGLB media (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)
Lauryl tryptose broth (Difco, BBL, Oxoid, Merck)
Desoxicolate agars (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)
Violet red bile salt media (Difco, BBL, Oxoid, Merck)
EC media (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)
EMB agars (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)
Endo's media (Difco, BBL, Oxoid, Merck)
Lactose broth (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)
IMViC test media
SIM media (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)
Glucose phosphate peptone media (Nissui, Eiken)
Simmons citrate media (Nissui, Eiken, Kyokuto, Difco, BBL, Oxoid, Merck)

(d) Reagents

Gram's solution
Kovac or Ehrlich indole reagent

Kovac's reagent		
Paradimethylamino benzaldehyde		5 g
Amyl alcohol		75 ml
Concentrated hydrochloric acid		25 ml
Ehrlich's reagent		
Paradimethylamino benzaldehyde		1 g
Absolute ethanol		95 ml
Concentrated hydrochloric acid		20 ml
Methyl red reagent		
Methyl red		0.5 g
Absolute ethanol		200 ml
VP reagent		
Reagent 1.	Alpha-naphthol	6 g
	Absolute ethanol	100 ml
Reagent 2.	40% potassium hydroxide solution	100 ml
	Creatine	0.3 g

(2) Sampling and sample preparation

Each food item and their environmental equipment (e.g., containers, cutting boards) with which the food is handled, are targeted for the tests. In general, focal coliform bacteria or E.coli tests are applied rather than coliform bacteria in unheated food, including raw meat, sea food and fresh vegetables, in which natural contamination is reflected as it is.

Sampling and preparation method of samples are common in general bacterial tests. Namely, samples are treated aseptically in order to avoid secondary contamination by microorganisms at the time of sampling, and food in a container or packaging is sampled as it is, in principle. For food in a large form or unpackaged, a piece should be sampled aseptically in a presterilized glass, polyethylene or plastic sampling container. In general, solid food is highly possibly to have dispersed contamination, therefore, 200g or more should be sampled from 5 different sites by using sterilized spoons or tweezers, and for liquid and powder foods, it should be mixed well and a portion should be sampled. For foods with ingredient standards, a quantity which can determine whether it matches the above or not should be sampled.

Samples should be kept at below 4 °C and examined within 4 hours after sampling, if the time exceeds 6 hours, it should be mentioned in the test result sheet.

Regarding the sample preparation, in general, liquid samples becomes the sample stock solution. For semisolids with high viscosity, powder and solid samples, weigh a certain quantity and add a 9-fold quantity of dilution water to make the sample stock solution. On this occasion, 25 g is weighed in principle however, 10 g is sufficient in cases where the distribution of bacteria is uniform. In the case of a smear material, sample stock solution is obtained from smear gauze, tampon, etc. which is then washed with a certain amount of dilution water. According to need, sample diluent should be prepared from sample stock solution with a 10-fold serial dilution by dilution water. If food ingredient standards are specified, sample solution should be prepared in accordance with the test method.

(3) Test methods

(a) Coliform bacteria

The procedures below should be applied.

Method with broth medium

Test methods include qualitative tests to find out the presence of 1 or more coliform bacteria in the sample, and quantitative tests to calculate the number of bacteria stochastically by most probable number (MPN). Qualitative test is generally applied to samples which are considered to have a low number of bacteria. In general, both procedures consist of 3 steps: estimation → definition → complete test, as shown in Figure 1-8..

1) Estimation test

There are 2 methods using BGLB medium and lactose broth medium depending on the test subject, and they both are performed in broth fermentation tubes with Durham tubes in them. In principle, BGLB medium is applied to all categories of foods rich in nutrition for bacteria, while lactose broth is applied to seawater fish and shellfish, industrial (agricultural) water, ice

/ snow, and smear materials such as food handling equipment poor in nutrition. Use of lauryl tryptose broth is widespread irrespective to the type of test targets in Europe and the United States.

In qualitative tests, generally the sample stock solution 10, 1 and 0.1 ml are inoculated to 2 broth fermentation tubes, respectively. However, in the case of inoculation of 10 ml, broth fermentation tubes with a 2-fold concentration are used in which 10 ml is infused.

In the quantitative test, sample stock solution 10 and 1 ml and 1 ml each of 10-fold serially diluted sample is inoculated to 5 or 3 broth fermentation tubes, respectively for each of 3 or greater successive steps according to the expected amount of bacteria. Sample-inoculated broth fermentation tubes are incubated for 24 ± 2 hours at 35 ± 1 °C, and if gas generation is noted it is considered as a positive estimation result, and then is proceeded to the step, the next definition test. If gas generation is not noted, incubation is continued up to 48 ± 3 hours and re-determined. As a result of re-determination, if gas generation is not noted, it is considered as coliform bacteria negative, and if gas generation is noted it is considered as a positive estimation result and conduct the next definition test.

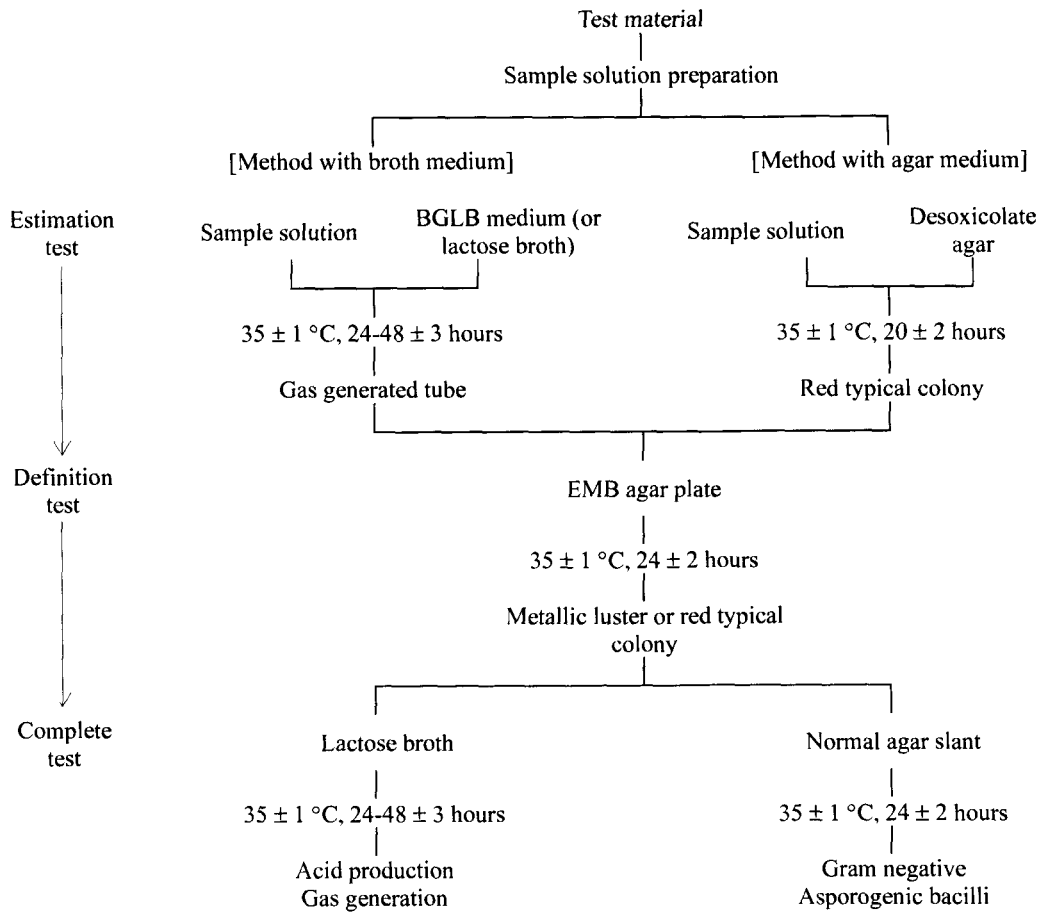

Figure 1-8 Test procedures of coliform bacteria

A
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2

L. plantarum viable count determination

1. Agar plate dilution method

Measure 1 g of sample precisely, add 9 mL of solvent to make it a 10-fold diluted suspension, and perform the following procedures.
Confirm that *L. plantarum* viable count is 100 counts/g or below.

(1) Pour 1 mL of the above 10-fold diluted suspension into three dishes.
(2) Add approximately 19 mL of SYP medium, which kept at 50°C after sterilization, to the dishes in (1) and mix gently.
(3) After the dishes in (2) are solidified, culture in an incubator at 32°C ± 1°C for 42 to 72 hours.
(4) Count the number of appeared colonies, and confirm that the average value of the three dishes is 10 or below.

2. Solvent

Potassium dihydrogenphosphate	4.5 g
Potassium hydrogenphosphate	5.0 g
Tween 80	0.5 g
L-cysteic hydrochloride hydrate	0.5 g
Agar	1.0 g
Deionized water	1000 mL

Use after heat sterilization at 121°C for 15 minutes by an autoclave.
(Dispense 9 mL of solvent to a test tube with an aluminum cap in advance)

3. SYP medium

Soluble starch	10.0 g
Yeast extract S	10.0 g
Polypeptone	5.0 g
Sodium acetate trihydrate	2.0 g
Tween 80	0.5 g
Salt solution*	5 mL
Agar	5.0 g
Deionized water	1000 mL

Use after heat sterilization at 121°C for 15 minutes by an autoclave.

*Salt solution (in 1 mL)

$MgSO_4$ hepatahydrate	40 mg
$MnSO_4$ pentahydrate	2 mg
$FeSO_4$ hepatahydrate	2 mg
NaCl	2 mg

Appendix C

APPENDIX C

SUMMARY OF ANALYTICAL DATA AND OTHER COMPOSITIONAL INFORMATION

Summary of Analytical Data and Other Compositional Information

Three (3) non-consecutive sample lots of the HK-LP ingredient (Lot Nos. 060621, 080524 and 081114) were analyzed to verify that the manufacturing process produces a consistent product. The analytical data show conformity to the product specifications and the absence of any chemical impurities or microbial contamination. The data also demonstrate that residual levels of the raw materials used as components of the culture medium are well below the specification parameters, demonstrating successful removal of these materials during the filtration process. For instance, bovine skim milk protein hydrolysate is a component of the medium used to culture *L. plantarum* L-137 in the production of the HK-LP ingredient. Therefore, the final ingredient was analyzed for lactose and the major known milk allergens, β-lactoglobulin and casein, in 3 non-consecutive lots. The analytical data demonstrate that these milk allergens are not present above specified limits in the final HK-LP ingredient. Lactose levels also were determined to be consistently below the limit specified. A summary of the physical, chemical, and microbiological product analyses for the 3 non-consecutive lots of the HK-LP ingredient is presented in Table C-1. The product specifications and certificates of analysis are included in Attachment C-1 and C-2, respectively.

The final ingredient also was analyzed for levels of protein and nucleic acid, resulting from the presence of HK-LP, in 3 non-consecutive lots, the analyses of which are presented in Table C-2. The average protein content in the HK-LP ingredient was determined to be 3.8%, which corresponds to an HK-LP protein content of approximately 20%. The average nucleic acid content was determined to be 2.4%, corresponding to an HK-LP nucleic acid content of 12%. In addition, the HK-LP ingredient was analyzed for the potentially harmful biogenic amines histamine and tyramine, which may be metabolically produced by lactobacilli. The analytical results are summarized in Table C-3 and demonstrate that neither histamine nor tyramine was identified in the final ingredient at detectable levels. The certificates of analysis and analytical results for these data are included in Attachment C-2.

Due to the potential development of antibiotic resistance among target bacterial species, microorganisms used in food should not produce antibiotic compounds that have similar mechanisms of action to clinically-important antibiotics. HWFC has measured the antibiotic activity of three non-consecutive lots of the HK-LP ingredient (Lot Nos. 060621, 080524 and 081114). The final ingredient did not exhibit antibiotic activity, and therefore, is not a concern in this regard and would not have implications in the development of resistance to bacteriocins. Please refer to the certificates of analysis in Attachment C-2.

Table C-1 Summary of Manufactured Lots of a Heat-Killed *Lactobacillus plantarum* Ingredient

Specification Parameter	Specification	Manufacturing Lot		
		Lot# 060621	Lot# 080524	Lot# 081114
Appearance	Light brown powder free of off-taste and off-flavor	Passed	Passed	Passed
Foreign substances	Negative	Negative	Negative	Negative
HK-LP Content, %	20±2	20.0	18.1	20.2
Dextrin content, %	76±6	77.2	78.3	77 5
Grain size (residue of #30), %	0	0	0	0
Bovine milk protein, µg/g	≤20	14.0	14 4	1.7
Casein, µg/g	≤10	1.4	≤ 1	≤ 1
β-Lactoglobulin, µg/g	≤10	≤ 1.0	2.4	≤ 1.0
Lactose, mg/g	≤2	≤ 2	≤ 2	≤ 2
Loss on drying, %	≤8	4.0	2.3	3.8
Residue on ignition, mg/g	≤30	24.4	16.9	18.2
Sodium acetate, µg/g	≤100	≤ 100	≤ 100	≤ 100
Manganese, µg/g	≤30	6	8	6
Lead, µg/g	≤ 1	≤ 0.05	≤ 0 05	≤ 0.05
Number of bacteria, CFU/g	≤ 1 x 10^3	0	5	29
Number of living *L. plantarum*, CFU/g	≤ 1 x 10^2	0	0	23
Salmonella species	Negative	ND	Negative	Negative
Coliforms (including *Escherichia coli*)	Negative	Negative	Negative	Negative
Yeast and mold, CFU/g	≤1 x 10^2	0	0	0

Abbreviations: As_2O_3 = arsenic oxide, CFU = colony-forming units; DE = dextrose equivalents; HK-LP = heat-killed *Lactobacillus plantarum*; ND = not determined; Pb = lead

Table C-2 Protein and Nucleic Acid Analysis of Manufactured Lots of a Heat-Killed *Lactobacillus plantarum* Ingredient

Parameter	Method of Analysis	Manufacturing Lot Numbers		
		Lot# 060621	Lot# 080524	Lot# 081114
Protein[a], g/100 g	BCA assay	3.6	4 2	3.6
Nucleic acid[a], g/100 g	Spectrophotometry	1.5	3.0	2.6
Histamine[b], mg/100 g	HPLC[c]	Not detected	Not detected	Not detected
Tyramine[b], mg/100 g	HPLC[c]	Not detected	Not detected	Not detected

Abbreviations: BCA = Bicinchoninic acid; HPLC = high-performance liquid chromatography
[a] Analytical results are included in Attachment C-2.
[b] Certificates of analysis are included in Attachment C-2.
[c] The limit of detection is 0.5 mg/100 g.

APPENDIX C
ATTACHMENT C-1
PRODUCT SPECIFICATIONS

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Specifications for LP20

Specification Parameter	Specification	Method of Analysis
Appearance	Light brown powder free of off-taste and off-flavor	Visual inspection and sensory test
Foreign substances	Negative	Visual inspection
HK-LP content	20±2%	Dry weight method for bacterial cell mass
Dextrin content	76±6%	Glucose oxidase-mutarotase method following HCl hydrolysis
Grain size, residue of #30	0%	Standard sieve method with sieve opening of 500 μm
Bovine milk protein	≤ 20 μg/g	ELISA
Casein	≤ 10 μg/g	ELISA
β-Lactoglobulin	≤ 10 μg/g	ELISA
Lactose	≤ 2 mg/g	HPLC method
Sodium acetate	≤ 100 μg/g	HPLC method
Loss on drying	≤ 8%	Loss on Drying test
Residue on ignition	≤ 30 mg/g	Residue on Ignition
Manganese	≤ 30 μg/g	ICP emission spectrometry
Heavy metal (as Pb)	≤ 20 μg/g	Heavy metals limit test
Lead	≤ 1 μg/g	Atomic absorption spectrophotometry
Arsenic (as As_2O_3)	≤ 2 μg/g	Arsenic limit test - Apparatus A
Number of mesophilic aerobic bacteria	≤ 1 x 10^3 CFU/g	Plate count method
Number of living *L. plantarum*	≤ 1 x 10^2 CFU/g	Viable count determination
Salmonella species	Negative	Isolation culture methods
Coliforms (including *Escherichia coli*)	Negative	Most probable number method
Yeast and mold	≤ 1 x 10^2 CFU/g	Dilution plating method

APPENDIX C
ATTACHMENT C-2
CERTIFICATES OF ANALYSIS AND OTHER ANALYTICAL DATA

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Certifications of analysis

Product Name	LP20	
Lot. No.	060621	
Specification Parameter	Specification	Results
Appearance	Light brown powder free of off-taste and off-flavor	Passed
Foreign substances	Negative	Negative
HK-LP content	20±2%	20.0
Dextrin content	76±6%	77.2
Grain size, residue of #30	0%	0
Bovine milk protein	≤ 20 μg/g	14.0
Casein	≤ 10 μg/g	1.4
β-Lactoglobulin	≤ 10 μg/g	≤ 1
Lactose	≤ 2 mg/g	≤ 2
Sodium acetate	≤ 100 μg/g	≤ 100
Loss on drying	≤ 8%	4.0
Residue on ignition	≤ 30 mg/g	24.4
Manganese	≤ 30 μg/g	6
Heavy metal (as Pb)	≤ 20 μg/g	≤ 20
Lead	≤ 1 μg/g	≤ 0.05
Arsenic (as As_2O_3)	≤ 2 μg/g	≤ 2
Number of mesophilic aerobic bacteria	≤ 1 x 10^3 CFU/g	0
Number of living *L. plantarum*	≤ 1 x 10^2 CFU/g	0
Salmonella species	Negative	-
Coliforms (including *Escherichia coli*)	Negative	Negative
Yeast and mold	≤ 1 x 10^2 CFU/g	0
Decision date	Overall conclusion	Certifier
January 5, 2010	Passed	International department General manager (b) (6)

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Certifications of analysis

Product Name	LP20	
Lot. No.	080524	
Specification Parameter	Specification	Results
Appearance	Light brown powder free of off-taste and off-flavor	Passed
Foreign substances	Negative	Negative
HK-LP content	20±2%	18.1
Dextrin content	76±6%	78.3
Grain size, residue of #30	0%	0
Bovine milk protein	≤ 20 μg/g	14.4
Casein	≤ 10 μg/g	≤ 1
β -Lactoglobulin	≤ 10 μg/g	2.4
Lactose	≤ 2 mg/g	≤ 2
Sodium acetate	≤ 100 μg/g	≤ 100
Loss on drying	≤ 8%	2.3
Residue on ignition	≤ 30 mg/g	16.9
Manganese	≤ 30 μg/g	8
Heavy metal (as Pb)	≤ 20 μg/g	≤ 20
Lead	≤ 1 μg/g	≤ 0.05
Arsenic (as As_2O_3)	≤ 2 μg/g	≤ 2
Number of mesophilic aerobic bacteria	≤ 1 x 10^3 CFU/g	5
Number of living *L. plantarum*	≤ 1 x 10^2 CFU/g	0
Salmonella species	Negative	Negative
Coliforms (including *Escherichia coli*)	Negative	Negative
Yeast and mold	≤ 1 x 10^2 CFU/g	0
Decision date	Overall conclusion	Certifier
January 5, 2010	Passed	International department General manager (b) (6)

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Certifications of analysis

Product Name	LP20	
Lot. No.	081114	
Specification Parameter	Specification	Results
Appearance	Light brown powder free of off-taste and off-flavor	Passed
Foreign substances	Negative	Negative
HK-LP content	20±2%	20.2
Dextrin content	76±6%	77.5
Grain size, residue of #30	0%	0
Bovine milk protein	≤ 20 μg/g	1.7
Casein	≤ 10 μg/g	≤ 1
β-Lactoglobulin	≤ 10 μg/g	≤ 1
Lactose	≤ 2 mg/g	≤ 2
Sodium acetate	≤ 100 μg/g	≤ 100
Loss on drying	≤ 8%	3.8
Residue on ignition	≤ 30 mg/g	18.2
Manganese	≤ 30 μg/g	6
Heavy metal (as Pb)	≤ 20 μg/g	≤ 20
Lead	≤ 1 μg/g	≤ 0.05
Arsenic (as As_2O_3)	≤ 2 μg/g	≤ 2
Number of mesophilic aerobic bacteria	≤ 1 x 10^3 CFU/g	29
Number of living *L. plantarum*	≤ 1 x 10^2 CFU/g	23
Salmonella species	Negative	Negative
Coliforms (including *Escherichia coli*)	Negative	Negative
Yeast and mold	≤ 1 x 10^2 CFU/g	0
Decision date	Overall conclusion	Certifier
January 5, 2010	Passed	International department General manager (b) (6)

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

April 12, 2009

Measurements of protein and nucleic acid

Contents of protein and nucleic acid have been measured for three non-consecutive lots of LP20 (Lot Nos. 060621, 080524 and 081114).

Measurement

Measurements of protein and nucleic acid in LP20 were carried out by our laboratory. Simplified measurement flow charts are shown on attached sheets.

Results

1) Protein

Name of sample	Protein (g/100 g)	Method
LP20 Lot 060621	3.6	BCA method
LP20 Lot 080524	4.2	BCA method
LP20 Lot 081114	3.6	BCA method

2) Nucleic acid

Name of sample	Nucleic acid (g/100 g)	Method
LP20 Lot 060621	1.5	Spectrophotometric method
LP20 Lot 080524	3.0	Spectrophotometric method
LP20 Lot 081114	2.6	Spectrophotometric method

Discussion

1) Protein

The average value of protein contents in LP20 is 3.8%, consequently protein contents in heat-killed *Lactobacillus plantarum* L-137 (HK-LP) is approximately 20%.

According to the "Applied microbiology, new edition" (1981, Asakura Publishing), protein contents in bacteria are 40% to 70% (Table 17).

We have measured contents of protein which was soluble under the pretreatment condition described in Attachment 1. In the case of the presence of a substantial amount of insoluble protein, protein contents in HK-LP may extend well beyond 20%. Thus the discrepancy between our measured value and the reported value may stem from the detection of insoluble protein.

Alternatively, heat treatment of *Lactobacillus plantarum* L-137 may cause protein leakage from bacterial cells. Even so, we can detect considerable amount soluble protein in HK-LP.

2) Nucleic acid

The average value of nucleic acid contents in LP20 is 2.4%, consequently nucleic acid contents in heat-killed *Lactobacillus plantarum* L-137 (HK-LP) is approximately 12%.

According to the "Applied microbiology, new edition" (1981, Asakura Publishing), nucleic acid) contents in bacteria are 15% to 25% (Table 17).

We have adopted the pretreatment condition suitable for extraction of double-stranded DNA. Extraction efficiency of single-stranded DNA, partially decomposed DNA or RNA is obscure by the method. If nucleic acid was not fully extracted, nucleic acid in HK-LP may extend well beyond 12%. Thus the marginal difference between our measured value and the reported value may stem from the pretreatment condition.

Alternatively, heat treatment of *Lactobacillus plantarum* L-137 may cause nucleic acid leakage from bacterial cells. Even so, we can detect appropriate amount of nucleic acid in HK-LP.

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Attachment 1

Simplified measurement flow chart for protein

Material sampling

| + distilled water

100 μl of suspended solution (10 mg/ml) *1

| + 25 μl of 100% trichloroacetic acid

Incubation on ice for 30 minuets

|

Centrifugation at 19000 g for 10 minuets at 4°C

|

Removal of supernatant

| + 100 of 0.1 N NaOH solution containing 5% sodium dodecyl sulfate

Resuspending pellet

|

Placing in an ultrasound bath for 5 minuets

|

Immersing in a boiling water for 10 minuets

|

Placing in an ultrasound bath for 5 minuets

|

Centrifugation at 19000 g for 5 minuets at 4°C

|

Isolation of supernatant fractions

|

Test solution

|

Bicinchoninic acid (BCA) method

*1: same procedures for bovine serum albumin (BSA) standard solution

House Wellness Foods Corporation
Imoji 3-20, Itami, Hyogo 664-0011, Japan

Attachment 2

Simplified measurement flow chart for nucleic acid

Material sampling

| + 500 μg/ml lysozyme solution in 100 mM Tris-HCI (pH 8.0)

1 ml of suspended solution (10 mg/ml)

|

Incubation at 37°C for 2 hours

| + 60 μl of 10% sodium dodecyl sulfate solution

| immixture

Incubation at 60°C for 10 minuets

|

Isolation of 500 μl

| + 500 μl of PCI solution (Tris-EDTA-saturated phenol: chloroform: isoamylalcohol in the ratio 25:24:1)

| immixture

Centrifugation at 19000 g for 10 minuets at room temperature

|

Isolation of 400 μl of supernatant fraction

| + 400 μl of isopropanol

| + 40 μl of 3 M sodium acetate

Incubation at -20°C for 20 minuets

|

Centrifugation at 19000 g for 20 minuets at 4°C

|

Removal of supernatant

|

Washing pellet with 70% ethanol

|

Allowing pellet to air-dry

|

Dissolving pellet with 1 ml of distilled water at 50°C

|

Dilution 10 times with distilled water

|

Test solution

|

Spectrophotometric method (Nucleic acid concentration is determined by measuring absorbance at 260 nm assuming that 1.0 unit of OD is equivalent to 50 μg/ml DNA)

Japan
Food
Research
Laboratories

No. 209021905-001
March 9, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20
Lot 060621

Japan Food Research Laboratories
Head Office: 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
Osaka Branch: 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
Nagoya Branch: 5-13 Osu 4-chome, Naka-ku, Nagoya 460-0011
Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on February 25th, 2009 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Histamine	Not detect	0.5 mg/100 g		HPLC method

Period.

000116

Japan
Food
Research
Laboratories

No. 209021905-002
March 9, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20
Lot 080524

Japan Food Research Laboratories
Head Office: 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
Osaka Branch: 3-1 Toyotsu-cho, Suita-shi, Osaka 564-0051
Nagoya Branch: 5-13 Osu 4-chome, Naka-ku, Nagoya 460-0011
Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on February 25th, 2009 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Histamine	Not detect	0.5 mg/100 g		HPLC method

Period.

000117

Japan
Food
Research
Laboratories

No. 209021905-003
March 9, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20
Lot 081114

Japan Food Research Laboratories

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Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on February 25th, 2009 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Histamine	Not detect	0.5 mg/100 g		HPLC method

Period.

Japan Food Research Laboratories

000118

Japan
Food
Research
Laboratories

No. 209021905-004
March 18, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20
Lot 060621

Japan Food Research Laboratories
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Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on February 25th, 2009 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Tyramine	Not detect	0.5 mg/100 g		HPLC method

Period.

Japan Food Research Laboratories

000119

Japan
Food
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Laboratories

No. 209021905-005
March 18, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20
Lot 080524

Japan Food Research Laboratories
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Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on February 25th, 2009 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Tyramine	Not detect	0.5 mg/100 g		HPLC method

Period.

Japan Food Research Laboratories

000120

Japan
Food
Research
Laboratories

No. 209021905-006
March 18, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20
Lot 081114

Japan Food Research Laboratories
Head Office: 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
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Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saitoasagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on February 25th, 2009 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Tyramine	Not detect	0.5 mg/100 g		HPLC method

Period.

000121

Japan
Food
Research
Laboratories

No. 208120873-005
January 6, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20 Lot 060621

Japan Food Research Laboratories
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Nagoya Branch: 5-13 Osu 4-chome, Naka-ku, Nagoya 460-0011
Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on December 9th, 2008 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Antibacterial activity	Negative		1	

Ref. 1. Appendix to Annex A, "Determination of antibiotic activity", in "Specifications for identity and purity of certain food additives" by Joint FAO/WHO Expert committee on Food Additives

Period.

Japan Food Research Laboratories

000122

Japan
Food
Research
Laboratories

No. 208120873-007
January 6, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20 Lot 080524

Japan Food Research Laboratories
Head Office: 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
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Nagoya Branch: 5-13 Osu 4-chome, Naka-ku, Nagoya 460-0011
Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on December 9th, 2008 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Antibacterial activity	Negative		1	

Ref. 1. Appendix to Annex A, "Determination of antibiotic activity", in "Specifications for identity and purity of certain food additives" by Joint FAO/WHO Expert committee on Food Additives

Period.

Japan Food Research Laboratories

000123

Japan
Food
Research
Laboratories

No. 208120873-008
January 6, 2009

Certificate of analysis

Client House Wellness Foods Corporation

Name of sample LP20 Lot 081114

Japan Food Research Laboratories
Head Office: 52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062
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Nagoya Branch: 5-13 Osu 4-chome, Naka-ku, Nagoya 460-0011
Kyushu Branch: 1-12 Shimogofuku-machi, Hakata-ku, Fukuoka-shi 812-0034
Tama Laboratory: 11-10 Nagayama 6-chome, Tama-shi, Tokyo 206-0025
Chitose Laboratory: 3 Bunkyo 2-chome, Chitose-shi, Hokkaido 066-0052
Saito Laboratory: 4-41 Saito-asagi 7-chome, Ibaraki-shi, Osaka 567-0085

Result of analytical test on the sample provided to us on December 9th, 2008 is as shown below.

Result of analytical test

Analytical test item	Result	Detection limit	Ref.	Method
Antibacterial activity	Negative		1	

Ref. 1. Appendix to Annex A, "Determination of antibiotic activity", in "Specifications for identity and purity of certain food additives" by Joint FAO/WHO Expert committee on Food Additives

Period.

Japan Food Research Laboratories

Appendix D

Appendix D

APPENDIX D

INFORMATION SUPPORTING THE STABILITY OF A HEAT-KILLED *LACTOBACILLUS PLANTARUM* (HK-LP) INGREDIENT

Information Supporting the Stability of a Heat-Killed *Lactobacillus plantarum* (HK-LP) Ingredient

Bulk Stability of HK-LP

The stability of the HK-LP ingredient is characterized by the stability of the HK-LP component, which was assessed through assays measuring the concentration of a fluorescent dye exclusively taken up by dead bacterial cells at various temperatures and durations. The number of stained (*i.e.*, dead), yet intact, HK-LP cells was measured by flow cytometry, which detects simultaneously the size (forward scatter) and shape (side scatter) of cells. Thus, intact HK-LP cells were identified by fluorescence intensity, size, and shape. From the relationship between the weight of HK-LP and the number of intact HK-LP cells, the amount of HK-LP in the final ingredient was determined.

The stability of HK-LP in the final ingredient is shown in Table D-1. The number of intact HK-LP cells was maintained following storage at 40°C for a period of 16 weeks, which empirically corresponds to approximately 2 years of storage at ambient temperatures. Therefore, HK-LP will remain stable within the final bulk ingredient at ambient temperatures for a period of up to at least 2 years as well as under elevated temperature conditions. Furthermore, from these data, the number of HK-LP cells per one gram of the final ingredient is an estimated 2.3×10^{11} cells. This number was used in subsequent stability experiments to predict the number of HK-LP cells present in various foods and beverages following the addition of the ingredient in specified amounts.

Table D-1 Stability of Heat-Killed *Lactobacillus plantarum* (HK-LP) in the Final HK-LP Ingredient Under Accelerated Conditions

Storage conditions		Number of Intact HK-LP Cells	
Temperature (°C)	**Time (weeks)**	**(cells/g)**	**(% Control)**
4 (control)	16	2.3×10^{11}	-
40	4	2.1×10^{11}	91
	8	2.2×10^{11}	96
	12	2.3×10^{11}	100
	16	2.2×10^{11}	96

HK-LP Stability in Select Food and Beverage Matrices

From the relationship between the weight of HK-LP and the number of intact HK-LP cells, the amount of HK-LP in the final ingredient or in proposed foods and beverages was determined. The stability of HK-LP in numerous food and beverage matrices under various conditions is presented below.

000127

The stability of HK-LP in select drink formulations with the added HK-LP ingredient is shown in Table D-2. The final ingredient was added to an isotonic drink formulation (pH 3.6) and to a fruit juice-containing soft drink (pH 2.7) at a concentration of 0.5 mg/mL, providing an estimated 1.15×10^8 HK-LP cells/mL. The number of intact HK-LP cells was determined to demonstrate stability following formulation and under various temperature conditions. The number of intact HK-LP cells remained stable during formulation of both drinks. HK-LP also was stable in the isotonic drink formulation under chilled conditions at 4°C and under accelerated conditions at 40°C for 4 weeks. Although HK-LP remained stable in the fruit juice-containing soft drink under chilled conditions, the number of intact HK-LP cells was substantially diminished under accelerated conditions. Therefore, the HK-LP ingredient would only be added to acidic drinks, with pH values of approximately 2.7 to 3.6, and when distributed under chilled conditions.

Table D-2 Stability of Heat-Killed *Lactobacillus plantarum* (HK-LP) in Select Drink Formulations

Storage Conditions	Number of Intact HK-LP Cells in an Isotonic Drink Formulation		Number of Intact HK-LP Cells in a Fruit Juice-Containing Soft Drink	
	(cells/mL)	(% Predicted Values)	(cells/mL)	(% Predicted Values)
12 weeks at 4°C	1.3×10^8	113	1.0×10^8	87
4 weeks at 40°C, followed by 8 weeks at 4°C	1.1×10^8	96	0.64×10^8	56

The stability of HK-LP in select foods with the added HK-LP ingredient is shown in Table D-3. The final ingredient was added to tofu at a concentration of 0.5 mg/g, providing an estimated 1.15×10^8 HK-LP cells/g. The number of intact HK-LP was reduced from this predicted amount during formulation and/or under storage at 4°C for a period of 12 weeks; however, the storage period of 12 weeks was longer than would be expected for a tofu product. Similarly, when the ingredient was added to chocolate at a concentration of 1 mg/g, providing an estimated 2.3×10^8 HK-LP cells/g, the number of intact HK-LP was lower than expected following storage under ambient conditions.

Table D-3 Stability of HK-LP in Selected Foods

Food	Storage conditions	Number of Intact HK-LP Cells	
		(cells/g)	(% Predicted Values)
Tofu	12 weeks at 4°C	0.60×10^8	52
Chocolate	12 weeks at ambient temperatures	1.5×10^8	65

The stability of HK-LP in an encapsulated product consisting of gelatin capsules containing cellulose powder, the HK-LP ingredient, calcium phosphate, and sucrose fatty acid ester is shown in Table D-4. The encapsulated product simulates low-moisture conditions. The HK-LP

ingredient was added to the encapsulated product at a concentration of 0.27 mg/mg, providing an estimated 6.3 x 10^7 HK-LP cells/mg or 6.3 x 10^{10} HK-LP cells/g. The number of intact HK-LP remained stable following storage at ambient temperatures over long periods of time. Therefore, the HK-LP ingredient may be added to low-moisture products that are distributed at ambient temperatures.

Table D-4 Stability of HK-LP in an Encapsulated Product

Storage conditions	Numbers of Intact HK-LP cells	
	(cells/g)	(% Predicted Values)
6 months at ambient temperatures	5.5×10^{10}	87
43 months at ambient temperatures	6.1×10^{10}	97

Based on the results of the studies evaluating the stability of intact HK-LP cells in the final HK-LP ingredient, as well as in select food and beverage matrices, the ingredient is stable under normal storage conditions as well as when added to acidic beverage formulations and powdered or low-moisture foods. When the HK-LP ingredient is added to high-moisture foods (*e.g.*, tofu), the storage conditions, including temperature and appropriate expiration dates, is determined for each individual food as the stability of the ingredient may vary with moisture.

SUBMISSION END

000130